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Work on development and/or validation of analytical methodologies during the current contract focused on assays for WR 6026 (and its metabolites, WR 211789 and WR 254421), mefloquine (and its metabolite, WR 160972), *p*-aminoheptanophenone (and related compounds), WR 242511, halofantrine (and its metabolite, WR 178,460, and their stereoisomers), chloroquine (and its metabolites, monodesethylchloroquine and didesethylchloroquine), WR 243,251, WR 238,605, gentamicin, paromomycin and artelinic acid (and metabolites and artesunate). Work on routine analyses of biological specimens during this period was performed for studies that required determination of concentrations of artelinic acid, chloroquine (and its metabolites, monodesethylchloroquine and didesethylchloroquine), and stereoisomers of halofantrine (and its metabolite, WR 178,460).

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INTRODUCTION

This report describes technical work accomplished and information gained in performance of contract number DAMD17-97-C-7058, titled "Analysis of Investigational Drugs in Biological Fluids - Method Development and Analysis of Pre-Clinical and Clinical Samples," for the US Army Medical Research and Development Command (USAMRDC). Using the experimental procedures described in this report, we maintain the capability to complete projects on up to one new compound (for which no method is described in the literature) and up to two compounds (for which methods are described in the literature) per year in terms of method development, validation, and characterization. We demonstrate sensitivity, specificity, linearity, lack of interferences, accuracy, and reproducibility of the analytical method, describe the extent of recovery for the method, and report on the stability of compounds of interest in specimens during storage and drug analysis. Validation of sensitive and specific analytical methods follow procedures described in the Analytical Section Procedural Manual, Procedure 2D-3.10, "Procedure for Validation" and earlier versions. Methods developed are such that a single technician can complete at least 15 clinical samples in one day. These methods are robust and portable enough to be transported to other laboratories. Within our routine analysis laboratory, we maintain the capability to assay up to 3,000 samples per year. Routine sample analysis will be performed in accordance with applicable procedures described in the Analytical Section Procedural Manual, Procedure 2D-4.6. "HPLC Run Setup" and Procedure 2D-10.4. "LC/MS/MS Run Setup" and earlier versions. We have sufficient equipment and personnel to develop several candidate agents simultaneously and to be able to respond to changing priorities. We prepare and submit required reports in accordance with the contracted schedule.

DISCUSSION

Purpose of the Present Work

Work on development and/or validation of analytical methodologies during the current contract focused on assays for WR 6026 (and its metabolites, WR 211789 and WR 254421), mefloquine (and its metabolite, WR 160972), *p*-aminoheptanophenone (and related compounds), WR 242511, halofantrine (and its metabolite, WR 178,460, and their stereoisomers), chloroquine (and its metabolites, monodesethylchloroquine and didesethylchloroquine), WR 243,251, WR 238,605, gentamicin, paromomycin and artelinic acid (and metabolites and artesunate). Work on routine analyses of biological specimens during this period was performed for studies that required determination of concentrations of artelinic acid, chloroquine (and its metabolites, monodesethylchloroquine and didesethylchloroquine), and stereoisomers of halofantrine (and its metabolite, WR 178,460).

For many years our research group has been actively involved in the development of analytical methods to assay for drug substances in biological fluids for pharmacokinetic, bioavailability, drug metabolism and drug monitoring studies. This report describes the approach we took to develop sensitive (picograms per milliliter of biological matrix), specific and quantitative analytical methods to support pharmacokinetic and bioavailability studies of candidate chemical warfare antidotes, antiparasitic drugs, radioprotectants and anti-infectious disease drugs.

In addition, routine analyses of biological specimens to support pharmacokinetic and bioavailability studies as part of preclinical and clinical investigations undertaken for the purpose of new drug development were performed as a significant adjunct to method development objectives. Within our routine analysis laboratory, we maintain the capability to assay up to 3,000 samples per year for this contract.

There are many reasons for the U.S. military to develop various new drugs to protect or to treat soldiers confronted with the hazards of the modern battlefield. Like any pharmaceutical company, however, the military has to provide documentation in support of Investigational New Drug (IND) submissions to the Food and Drug Administration (FDA). Therefore, a great deal of work involving animal studies, preclinical and clinical trials, toxicity, metabolism and formulations must be carried out before a drug can be tried in the field. All of these studies depend on the adequacy of the analytical method for the particular compound. The route of administration and the dosage form are not necessarily the same in the field as in the clinic. For example, pyridostigmine is given prophylactically in the field, but the dose and route of administration are different for the treatment of myasthenia gravis or in anesthesiology.[1] Since military personnel are constantly involved in areas where they can be infected by parasites, including tropical or subtropical zones with drug-resistant forms, the U.S. Army needs to organize programs so that highly active and more effective new drugs can be discovered. These types of programs are generally ignored by private industry due to limited markets and profits.

This contract has offered us an interesting and stimulating challenge to utilize and extend our considerable capabilities to conduct method development and routine analysis in support of pharmacokinetic and bioavailability studies. Our participation in this contract was possible by virtue of the experience and expertise of our staff in the area of pharmacokinetics, which requires assurance of extensive and rigorous internal and external analytical quality. As a result of our extensive involvement in these analytical programs, the staff members working on this project are the best in the field and have acquired a broad range of experience in the analysis of organic compounds in diverse media.

Background of Previous Work

Studies conducted over the first 3 years of contract DAMD17-97-C-7058 and the 13 prior years under previous contracts including DAMD17-92-C-2028,

DAMD17-86-C-6150, DAMD17-85-D-0008, and DAMD17-83-C-3004 are listed in Tables 1 (study reports) and 2 (routine analyses reports).

TABLE 1: PREVIOUS STUDIES

Report No.	Report Date	Report Title	Test System	Test Article	Lower Limit of Quantitation
01	8/26/83	Analytical Procedure for the Determination of WR 6026 in Plasma	plasma plasma blood blood	WR 6026 WR211,789•2HCl WR 6026 WR211,789•2HCl	6.44 ng/ml 8.00 ng/ml 6.44 ng/ml 8.00 ng/ml
03	1/22/85	High Pressure Liquid Chromatography (HPLC) of Pyridostigmine in Plasma	plasma	Pyridostigmine	1.4 ng/ml
04	8/23/85	Ion-Paired Liquid Chromatographic Method for the Analysis of Halofantrine (WR 171,669) and its Putative Metabolite, WR 178,460, in Blood and Plasma	plasma plasma blood blood	halofantrine WR 178,460 halofantrine WR 178,460	0.900 ng/ml 1.40 ng/ml 0.900 ng/ml 1.40 ng/ml
05	7/21/86	High Pressure Liquid Chromatography (HPLC) of Pyridostigmine in Plasma Using Silica Gel Column and an Aqueous Mobile Phase	plasma	Pyridostigmine	1.39 ng/ml
06	1/8/88	High Pressure Liquid Chromatography (HPLC) of Mefloquine in Plasma	plasma	Mefloquine	10.0 ng/ml
07	1/12/88	High Pressure Liquid Chromatography (HPLC) of Pyridostigmine in Urine	urine	Pyridostigmine	13.7 ng/ml
08	9/23/88	High Pressure Liquid Chromatography (HPLC) of Physostigmine in Plasma with Ultraviolet Detection	plasma	Physostigmine	1 ng/ml
09	9/12/88	Quantitation of Physostigmine & Eseroline in Plasma by HPLC with Fluorescence Detection	plasma plasma	Physostigmine eseroline	0.1 ng/ml 0.1 ng/ml
10	9/14/89	Quantitation of WR 6026 (Free Base) in Plasma & Blood by HPLC	plasma blood	WR 6026 WR 6026	0.980 ng/ml
11	9/28/89	Quantitation of WR 2721 in Plasma by HPLC with Electrochemical Detection	plasma	WR 2721	0.100 µg/ml

TABLE 1: PREVIOUS STUDIES

Report No.	Report Date	Report Title	Test System	Test Article	Lower Limit of Quantitation
12	11/14/89	Quantitation of WR 3689 in Plasma by HPLC with Electrochemical Detection	plasma	WR 3689	0.0990 µg/ml
13	11/17/89	Quantitation of WR 238605 by HPLC	plasma blood	WR 238,605 WR 238,605	0.815 ng/ml 1.91 ng/ml
13	10/28/94 final report	Supplement I: Quantitation of WR 238605 as Free Base in Rat Plasma by HPLC and Fluorescence Detection	rat plasma	WR 238,605	2.00 ng/ml
13	5/16/96 (accepted as final 3/3/98)	Supplement II: Quantitation of WR 238605 as Free Base in Dog Plasma by HPLC and Fluorescence Detection	dog plasma	WR 238,605	1.00 ng/ml
14	8/29/89	Quantitation of Mefloquine (f.b.) in Plasma by HPLC, Extract. Meth	plasma	Mefloquine	8.00 ng/ml
15	12/19/90	Quantitation of Ribavirin and WR 249,992 (f. b.) in Plasma by HPLC with C18 Bonded Silica Gel Columns and Acidic Aqueous Mobile Phases	plasma plasma	Ribavirin WR 249,992	20 ng/ml 10 ng/ml
16	Canceled	_-arteether project canceled		WR 255663	
17A	4/25/90 final	Quantitation of Halofantrine and WR 178,460 (as Free Bases) in Plasma and Blood by HPLC with a Silica Gel Column and an Aqueous Mobile Phase	human plasma plasma blood blood	halofantrine WR 178,460 halofantrine WR 178,460	0.960 ng/ml 0.928 ng/ml 0.960 ng/ml 0.928 ng/ml
17B	12/13/95 final as amended 4/26/96	Quantitation of Halofantrine and WR 178,460 (as Free Bases) in Plasma and Blood by HPLC with a Silica Gel Column and an Aqueous Mobile Phase	human plasma plasma blood blood	halofantrine WR 178,460 halofantrine WR 178,460	2 ng/ml 2 ng/ml 0.960 ng/ml 0.928 ng/ml
17B	1/23/98 final report	Supplement I: Quantitation of Halofantrine and WR 178,460 (as Free Bases) in Rat Perfusate by Precipitation and HPLC with a Silica Gel Column and an Aqueous Mobile Phase	rat perfusate	Halofantrine WR178460	0.520 µg/ml 0.510 µg/ml

TABLE 1: PREVIOUS STUDIES

Report No.	Report Date	Report Title	Test System	Test Article	Lower Limit of Quantitation
17B	1/28/98 final report	Supplement II: Quantitation of Halofantrine and WR 178,460 (as Free Bases) in Rat Perfusate by Extraction and HPLC with a Silica Gel Column and an Aqueous Mobile Phase	rat perfusate	Halofantrine WR178460	10.4 ng/ml 10.2 ng/ml
17B	1/28/98 final report	Supplement III: Quantitation of Halofantrine and WR 178,460 (as Free Bases) in Rat Bile by Precipitation and HPLC with a Silica Gel Column and an Aqueous Mobile Phase	rat bile	Halofantrine WR178460	0.416 µg/ml 0.408 µg/ml
17B	1/28/98 final report	Supplement IV: Quantitation of Halofantrine and WR 178,460 (as Free Bases) in Rat Bile by Extraction and HPLC with a Silica Gel Column and an Aqueous Mobile Phase	rat bile	Halofantrine WR178460	20.4 ng/ml
17B	1/28/98 final report	Supplement V: Quantitation of Halofantrine and WR 178,460 (as Free Bases) in Rat Liver by Precipitation and HPLC with a Silica Gel Column and an Aqueous Mobile Phase	rat liver	Halofantrine WR178460	0.540 µg/ml 0.540 µg/ml
18	Status Report 7/31/91	Quantitation of WR 6026 and WR 211,789 (WR 6026 Metabolite) in Plasma and Blood by HPLC with a Silica Gel Column and an Aqueous Mobile Phase	Plasma blood	WR 6026 WR 211789 WR 6026 WR 211789	0.980 ng/ml 1.21 ng/ml 0.980 ng/ml 1.21 ng/ml
19	Status Report 1/14/92	Quantitation of Mefloquine in Human Blood By HPLC, Extraction Method	human blood	mefloquine WR 160972	7.36 ng/ml ng/ml
20	7/27/94 final report	Quantitation of Artelinic acid in Plasma by HPLC with a C18 Bonded Column	human plasma	Artelinic Acid	4.96 ng/ml
23	4/29/96 final report	Quantitation of Primaquine (Free Base) and its Carboxylated Metabolite in Human Plasma by HPLC and Ultraviolet Detection	human plasma	Primaquine WR 249725	28.5 ng/ml 20.0 ng/ml

TABLE 1: PREVIOUS STUDIES

Report No.	Report Date	Report Title	Test System	Test Article	Lower Limit of Quantitation
24	10/21/97 final report	Quantitation of Paromomycin and Gentamicin in Human and Rat Plasma by HPLC	human plasma rat plasma	Gentamicin Paromomycin Gentamicin Paromomycin	0.1 µg/ml 0.1 µg/ml 0.1 µg/ml 0.1 µg/ml
25	11/22/95 final report amended 3/29/96	Quantitation of Pyridostigmine (as Free Base) in Human Plasma by HPLC w/ Silica Gel Column and an Aqueous Mobile Phase	human plasma	Pyridostigmine	1.53 ng/ml
26	12/12/96 final report, amend. in preparat'n	Quantitation of WR 242511 (as Free Base) in Human and Dog Plasma By HPLC with a Silica Gel Column and an Aqueous Mobile Phase	human plasma dog plasma	WR 242511 WR 242511	4.00 ng/ml 4.00 ng/ml
27	12/17/97 final report	Quantitation of WR 238605 *R&S* Enantiomers (as Free Bases) in Human Plasma by HPLC	human plasma	*R* WR 238605 *S* WR 238605	5 ng/ml 5 ng/ml
31 part 1	May 10, 1999 final report	Validation of LC/MS/MS Method for the Determination of Chloroquine (& its metabolites), Quinine, Doxycycline, Halofantrine (& its metabolite), Mefloquine, and WR 238,605 in Dog Plasma Samples, Part I: WR 238,605 and Mefloquine	dog plasma	WR 238605 Mefloquine	4.00 ng/ml 5.00 ng/ml
31 part 2	May 13, 1999 final report	Part II: Chloroquine and Quinine	dog plasma	Chloroquine (C) MonodesethylC DidesethylC Quinine	4.00 ng/ml 4.00 ng/ml 4.00 ng/ml 10.0 ng/ml
31 part 3	April 21, 2000 final report	Part III: Doxycycline	dog plasma	Doxycycline	50.0 ng/ml
31 part 4	May 1, 2000 final report	Part IV: Halofantrine (and its metabolite) and WR 238,605	dog plasma	Halofantrine WR 178,460 WR 238,605	2.00 ng/ml 2.00 ng/ml 2.00 ng/ml
35	March 29, 2000 final report	LC/MS/MS Method for the Determination of Artelinic Acid in Dog Plasma Samples	dog plasma	Artelinic Acid	4.00 ng/ml

TABLE 2: PREVIOUS ROUTINE ANALYSES PERFORMED

Report Title	Report Date	Test Article	Test System	No. of Samples	Report No.
Routine Analysis of Pyridostigmine Plasma Samples Obtained from Protocol Titled "Pharmacokinetics of Orally Administered Pyridostigmine and Comparative Bioavailability of Liquid and Tablet Formulations" (Subjects 1-30)	12/3/86	Pyridostigmine Pyridostigmine	plasma dose sol	1698 12	PY 85-1
Routine Analysis of Pyridostigmine Plasma Samples Obtained from Protocol Titled "14 day pilot dose range oral toxicity study in dogs" (Battelle)	7/30/87	Pyridostigmine Pyridostigmine	plasma dose sol	152 2	PY 85-2-2B
Routine Analysis of Pyridostigmine Plasma Samples from Battelle Laboratories-MREF Protocol 27 (Battelle)	7/9/86	Pyridostigmine Pyridostigmine	plasmaplasma,blind	648 22	PY 85-2-3
Routine Analysis of Pyridostigmine Plasma Samples obtained from Protocol Titled "Development of a Primate Model for Evaluating Efficacy of Treatment Regimens Against Nerve Agent Poisoning:Part I: Pharmacokinetics of Pralidoxime Chloride, Atropine Sulfate, and Pyridostigmine Bromide" (PY85-3-1 through PY85-3-5 combined)	5/29/87	Pyridostigmine	plasma, monkey	439	PY 85-3-6B
Pyridostigmine in plasma (Israel)	5/14/86	Pyridostigmine	plasma	427	PY 85-4
Routine Analysis of Pyridostigmine Plasma Samples obtained from Protocol titled "Comparative Bioavailability Studies of Pyridostigmine Bromide in Male Beagle Dogs " (31 July 1985) (Huntingdon dog)	10/7/87	Pyridostigmine	plasma, dog	324	PY 85-5C

TABLE 2: PREVIOUS ROUTINE ANALYSES PERFORMED

Report Title	Report Date	Test Article	Test System	No. of Samples	Report No.
Pyridostigmine in plasma (Huntingdon dog)	9/30/87	Pyridostigmine	plasmad og	336	PY 85-5-3C
"Pyridostigmine in plasma" (PY85-6-2 and PY85-6-3 combined) (Johns Hopkins, Millers)	7/3/86	Pyridostigmine	plasma	32	PY 85-6-4
"Pyridostigmine in plasma (Johns Hopkins,Sub.1-24)"	1/12/87	Pyridostigmine pyridostigmine	plasma infusate	969 23	PY 85-6-5
"Pyridostigmine in plasma (Johns Hopkins,Sub.1-24)"	3/12/87	Pyridostigmine Pyridostigmine	plasma dose sol	1102 27	PY 85-6-6B
Battelle Rat Study Pyridostigmine in plasma (revised letter report)	7/28/87	Pyridostigmine	plasma, rat	102	none none
Battelle Dosing Sol'ns Pyridostigmine in plasma (revised letter report)	7/28/87	Pyridostigmine	dose sol	92	none none
Routine Analysis of Halofantrine Plasma Samples Obtained from Protocol Titled "The Relative Bioavailability of Three Oral Formulations of Halofantrine Hydrochloride"	10/23/87	Halofantrine WR 178,460	plasma plasma	971 971	AY 86-1D
Routine Analysis of WR 6026 Plasma Samples Obtained from Clinical Protocol Titled "Single-Dose Absorption and Pharmacokinetics of WR 6026 Hydrochloride in Healthy Subjects"	6/24/87	WR 6026	plasma	192	AY 86-2D
Routine Analysis of Pyridostigmine Urine Samples from Protocol Titled "Bioavailability of Oral Pyridostigmine and Inhibition of Red Blood Cell Acetylcholinesterase by Oral and Intravenous Pyridostigmine"	2/3/88	Pyridostigmine	urine	110	Pyr/U (renamed from AY86-3) 86-3B

TABLE 2: PREVIOUS ROUTINE ANALYSES PERFORMED

Report Title	Report Date	Test Article	Test System	No. of Samples	Report No.
Routine Analysis of Mefloquine Plasma Samples obtained from Six Clinical Protocols from Thailand	2/25/88	Mefloquine	plasma	781	Mef/P 87-1B
Routine Analysis of Pyridostigmine Plasma and Urine Samples from Protocol Titled "Pharmacokinetics and Pharmacodynamics of Sustained, Low-dose, Intravenous Infusions of Pyridostigmine"	2/24/88	Pyridostigmine Pyridostigmine Pyridostigmine	plasma urine infusate	498 72 24	Pyr/PU 87-2B
Routine Analysis of Pyridostigmine Plasma Samples from the Protocol titled "Comparative Oral Bioavailability Studies of Two Wax Matrix Formulations of Pyridostig- mine Bromide in Male Beagle Dogs"	3/29/88	Pyridostigmine	plasma	341	Pyr/P 88-1
Routine Analysis of Pyridostigmine Plasma Samples from the Protocol titled "Safety, Tolerance, Pharmacokinetics and Pharmaco- dynamics of Single Oral Doses of Sustained Release Pyridostigmine in Healthy Men," dated 9/18/87	8/3/88	Pyridostigmine	plasma	558	Pyr/P 88-2 &4
"Routine Analysis of Pyridostigmine Plasma Samples from the Protocol titled ""Safety, Tolerance, Pharmacokinetics and Pharmaco- dynamics of Single Oral Doses of Sustained Release Pyridostigmine in Healthy Men,"" dated Sept. 30, 1987"	8/2/88	Pyridostigmine	plasma	476	Pyr/P 88-3
Routine Analysis of Physostigmine Plasma Samples from the Protocol Titled "Bioavail-ability and Pharmacokinetic Study of Physostigmine (WR 006570) in Beagle Dogs"	8/26/88	Physostigmine Eseroline	plasma plasma	198 198	Phy/P 88-5

TABLE 2: PREVIOUS ROUTINE ANALYSES PERFORMED

Report Title	Report Date	Test Article	Test System	No. of Samples	Report No.
Routine Analysis of Physostigmine Plasma Samples from the Protocol Titled "Bioavailability and Pharmacokinetic Study of Physostigmine (WR 006570) in Rhesus Macaques"	9/15/88	Physostigmine Eseroline	plasma plasma	196 196	Phy/P 88-6
Routine Analysis of Blood Samples from the Protocol Titled "Multiple-Dose Pharmacokinetics, Safety and Tolerance of WR 6026 Hydrochloride in Healthy Subjects"	4/21/89	WR 6026	blood	571	Wr6/B 88-7
Pilot Study - Analysis of Rat Plasma	9/14/88	Physostigmine Eseroline	plasma plasma	45 45	Phy/rP 88-8
Pilot Study - Analysis of Rat Perfusate	9/14/88	Physostigmine	perfus	37	Phy/rPr, pilot 88-9
Pilot Study - Analysis of Monkey Plasma	5/5/88	Physostigmine Eseroline	plasma plasma	8 8	Phy/mP, pilot 88-10
Routine Analysis of Plasma Samples from Thailand for Mefloquine Concentrations	12/7/88	Mefloquine	plasma	388	Mef/P 88-11
Routine Analysis for protocol titled "Simultaneous Modeling of WR238605 Succinate Pharmacokinetics and Methhemoglobin Pharmacodynamics in the Beagle Dog"	4/13/89	WR 238605 WR 238605	plasma blood	62 62	WR5/BP, pilot 89-1
Routine Analysis for Protocol Titled "Safety, Tolerance, Pharmacokinetics and Pharmacodynamics of Single Oral Doses of Pyridostigmine Administered by an Osmotic-Delivery Module (osmetr) compared to Pyridostigmine Syrup in Healthy Men"	5/12/89	Pyridostigmine	plasma	374	Pyr/P 89-2

TABLE 2: PREVIOUS ROUTINE ANALYSES PERFORMED

Report Title	Report Date	Test Article	Test System	No. of Samples	Report No.
Routine Analysis for protocol titled "Safety, Tolerance, Pharmacokinetics and Pharmaco dynamics of Single Oral Doses of a Commercial Formulation of Sustained-Release Pyridostigmine in Healthy Men."	5/16/89	Pyridostigmine	plasma	120	Pyr/P 89-3
Routine Analysis for protocol titled "Simultaneous Modeling of WR238605 Succinate Pharmacokinetics and Methhemoglobin Pharmacodynamics in the Beagle Dog"	6/1/89	WR 238605 WR 238605	plasma blood	88 88	WR5/BP, pilot 89-4
Routine Analysis for protocol titled "Simultaneous Modeling of WR238605 Succinate Pharmacokinetics and Methhemoglobin Pharmacodynamics in the Beagle Dog"	8/25/89	WR 238605 WR 238605	plasma blood	240 240	WR5/BP 89-5
Routine Analysis of Physostigmine (f.b.) and Eseroline (f.b.) Rat Plasma Bile, and Tube Binding Samples for Samples Obtained from WRAIR	1/18/90	Physostigmine Eseroline Physostigmine Eseroline	plasma plasma bile etc bile etc	92 92 20 20	Phy/rP, pilot 89-6
Phase III Comparative Clinical Trial of 4 Regimens of Halofantrine and Chloroquine in Treatment of *P. falciparum* Malaria	6/27/90	Halofantrine WR 178,460 Halofantrine WR 178,460	plasma plasma blood blood	470 470 468 468	Hal/BP 89-7
Safety, Tolerance, Pharmacokinetics and Pharmacodynamics of Intravenous Pyridostigmine and Oral Doses of Standard and Sustained-Release Pyridostigmine in Healthy Men & the Influence of Food on Oral Pyridostigmine Pharmacokinetics	11/13/90	Pyridostigmine	plasma	1250	Pyr/P 89-8
Routine Analysis for Protocol Titled "Effect of chronic pyridostigmine administration on heavy exercise in hot environments"	9/11/90	Pyridostigmine	plasma	37	Pyr/P 90-2

TABLE 2: PREVIOUS ROUTINE ANALYSES PERFORMED

Report Title	Report Date	Test Article	Test System	No. of Samples	Report No.
Routine Analysis of Blood for Mefloquine (fb) Concentrations	2/12/91	Mefloquine	blood	18	Mef/B 90-3
Routine Analysis for Protocol Titled "Effects of Pyridostigmine Pretreatment on Physiological Responses to Heat & Moderate-to Intense Exercise"	2/20/91	Pyridostigmine	plasma	142	Pyr/P 90-4
Routine Analysis for Protocol Titled "Pharmacokinetics of Intravenous Halofantrine HCl"	12/18/90	Halofantrine WR 178,460 Halofantrine WR 178,460	plasma plasma blood blood sol'ns	434 434 429 429 20	Hal/PB 90-5 &1
Routine Analysis for WR 6026 and WR 211,789 (as f.b.) of Plasma Samples Obtained from WRAIR	2/13/91	WR 6026 WR 211789	plasma plasma	13 13	Wr6/PB 90-6
Routine Analysis for Halofantrine and WR 178,460 (as Free Bases) of Plasma Samples Obtained under the Protocol Titled "52-Week Chronic Oral Toxicity Study of WR 171,669 HCl (Halofantrine Hydrochloride) in Dogs" and "Analysis of Blood and Plasma to Verify in vitro Metabolism of Halofantrine and Partition of Halofantrine and WR 178,460"	7/16/91	Halofantrine WR 178,460 Halofantrine WR 178,460	plasma plasma blood blood	83 83 48 48	Hal/P 91-1&2
Routine Analysis of Plasma and Blood Samples for the Protocol Titled 'Disposition Kinetics of IV Desbutyl Halo-fantrine and the Effects of Gastric pH on the Bioavail-ability of Halofantrine-HCl'	2/4/92	Halofantrine WR 178,460 Halofantrine WR 178,460	plasma plasma blood blood dosing sol'ns	756 756 754 754 18	Hal/BP 91-3
Routine Analysis for Halofantrine and WR 178,460 (as free bases) of Plasma Samples Obtained for the Initial Year of the Protocol Titled "Combined Chronic Toxicity and Oncogenicity Study of WR-171,669 (Halofantrine Hydrochloride) in Rats"	3/31/93 final report	halofantrine WR 178,460	rat plasma	118 118	Hal/P 91-4

TABLE 2: PREVIOUS ROUTINE ANALYSES PERFORMED

Report Title	Report Date	Test Article	Test System	No. of Samples	Report No.
Routine Analysis for Halofantrine and WR 178,460 (free bases) in Blood Samples Obtained for the Protocol Titled "Efficacy of Halofantrine and Mefloquine in the Treatment of Falciparum Malaria"	1/21/92 final report	Halofantrine WR 178,460	human blood	107 107	Hal/B 91-5
Routine Analysis for Halofantrine and WR 178,460 (as free bases) of Blood Samples Obtained under the Protocol Titled "Efficacy of Halofantrine and Mefloquine in the Treatment of Falciparum Malaria"	6/23/92 final report	mefloquine	human blood	107	Mef/B 91-5
Results assoc. with Hal/P 91-1	4/28/92 final report	halofantrine WR 178,460	dog plasma	29 29	Hal/P 91-6
Routine Analysis for Mefloquine (as Free Base) in Plasma Samples Obtained under the Protocol Titled "Evaluation of the Tolerance of Prophylactic Mefloquine Regimens"	3/1/93 final report	mefloquine	human plasma	660	Mef/P 91-7
Study continued as WR6/PU 93-1	8/3/92 data	WR 6026 WR 211,789	plasma	194 194	WR6/P 92-1
Routine Analysis for Halofantrine and WR 178,460 (as f.b.) of Plasma Samples Obtained for the Second Year of the Protocol Titled "Combined Chronic Toxicity and Oncogenicity Study of WR-171,669 HCl (Halofantrine Hydrochloride) in Rats, HWA Study No. 193-558"	3/31/93 final report	halofantrine WR 178,460	rat plasma	154 154	Hal/P 92-2
Routine Analysis for WR 238,605 (as f.b.) of Blood and Plasma Samples Obtained for the Protocol Titled "Rising Single Oral Dose Safety and Tolerance Study of WR 238,605 Succinate"	2/6/95 final report	WR 238,605	human plasma, blood	893 74	WR5/PB 92-3

TABLE 2: PREVIOUS ROUTINE ANALYSES PERFORMED

Report Title	Report Date	Test Article	Test System	No. of Samples	Report No.
Routine Analysis for WR 6026, WR 211,789 and WR 254,421 (as free bases) in Plasma and Urine Samples Obtained under the Protocol Titled "Phase II Clinical Trial of Oral WR 6026 2HCl in Patients with Vis-ceral Leishmaniasis - Initial Dose Ranging for Efficacy, Safety and Tolerance"	3/12/93 data	WR 6026 WR 6026 WR 211,789 WR 254,421	human plasma, urine	117 68 68 68	WR6/PU 93-1
Routine Analysis for WR 238,605 (as f.b.) of Plasma Samples Obtained for the Protocol Titled "Thirteen Week Oral Toxicity Study of WR 238,605 with a Thirteen Week Recovery Period in Dogs"	9/16/98 final report	WR 238,605	dog plasma	330	WR5/P 93-4
Routine Analysis for WR 238, 605 (as f.b.) of Plasma Samples Obtained for the Protocol Titled "Thirteen Week Oral Toxicity Study of WR 238,605 with a Thirteen Week Recovery Period in Rats"	1/20/94 final report amended 11/4/96 accepted as final 7/27/98	WR 238,605	rat plasma	154	WR5/P 93-5
Routine Analysis for Prima-quine and Carboxyprimaquine of Serum Samples Obtained for the Protocol Titled "Prima-quine and Several Recommended Prophylactic Drugs against Falciparum Malaria: Field Trial II"	5/3/96 final report	primaquine carboxy metab	human serum	60	Pri/P 93-6
Blind sample results to be added to SR 13B, Supplement II	10/12/94 final data	WR 238,605	dog plasma	30	WR5/P 94-5
Routine Analysis for Pyridostigmine (Cation) in Plasma Samples for the Protocol Titled "A Study to Evaluate the Safety, Tolerance, Pharmacokinetics and Pharmacodynamics of Pyridostigmine when given in Single and Multiple Doses to Males and Females in Diff	4/3/96 final report	Pyridostigmine	human plasma	2639	Pyr/P 94-6

TABLE 2: PREVIOUS ROUTINE ANALYSES PERFORMED

Report Title	Report Date	Test Article	Test System	No. of Samples	Report No.
Routine Analysis for WR 238605 in Plasma Samples Obtained for the Protocol Titled "A Multiple Dose Safety, Tolerance and Pharmacokinetic Study of WR 238605 when Given to Healthy Male and Female Subjects"	8/27/98 final report	WR 238605	human plasma	709	WR5/P 94-7
Routine Analysis for WR 238605 in Rat Plasma Samples Obtained for the Protocol Titled "Six Month Oral Toxicity Study of WR 238605 Succinate in Rats"	7/24/98 final report	WR 238605	rat plasma	405	WR5/P 95-1
Routine Analysis for WR 238,605 (f.b.) of Human Plasma and Blood Samples and for Chloroquine and Chloroquine Metabolite of Human Blood Samples Obtained for the Protocol Titled "Evaluation of WR 238605 as a Prophylactic Agent against Induced *P. falciparum* Malaria Infection in Healthy Non-Immune Subjects II: A Multiple-Dose Causal vs. Suppressive Study"	8/28/98 final report	WR 238605 chloroquine monodesethyl chloroquine	human plasma blood blood blood	226 226 67 67	WR5/P 95-2
Routine Analysis for the R andS Isomers of WR 238,605 (f.b.) of Human Plasma Samples Obtained for the Protocol Titled "Evaluation of WR 238605 as a Prophylactic Agent against Induced *P. falciparum* Malaria Infection in Healthy Non-Immune Subjects II: A Multiple-Dose Causal versus Suppressive Study"	9/2/98 final report	*R* WR 238605 *S* WR 238605	human plasma	226 226	WR5/P 95-2
Routine Analysis for Halo-fantrine and WR 178460 in Aotus Monkey Blood Samples	1/23/98 final report	Halofantrine	monkey blood	165	Hal/B 96-1

TABLE 2: PREVIOUS ROUTINE ANALYSES PERFORMED

Report Title	Report Date	Test Article	Test System	No. of Samples	Report No.
Routine Analysis for Gentamicin/Paromomycin (as f.b.) of Human Plasma Samples Obtained for the Protocol Titled "Irritant and Phototoxicity Reactions to the Topical Anti-leischmanial WR 279,369: A Randomized, Double-Blind Phase I Study"	8/9/00 final report	Gentamicin Paromomycin	human plasma	36 47	Gnt/P 96-3
LC/MS/MS Analysis for the Determination of Artelinic Acid in Dog Plasma Samples Obtained under Protocol 7869.14.01 (Task Order SR99-1) Titled "Effect of Artelinic Acid on Dogs after Oral Administration for 14 Days"	4/14/00 final report	Artelinic acid	dog plasma	244	Art/dp 99-1

Methods of Approach

The general development plan is described with emphasis on the laboratory procedures used. We worked on demonstrating sensitivity, specificity, linearity, lack of interferences, accuracy, and reproducibility of the analytical method, describing the extent of recovery for the method, and reporting on the stability of compounds of interest in specimens during storage and drug analysis. Validation of sensitive and specific analytical methods follow procedures described in the Analytical Section Procedural Manual, Procedure 2D-3.10, "Procedure for Validation of an Assay Methodology" and earlier versions. Methods are developed such that a single technician could complete at least 15 clinical samples in one day. These methods are to be robust and portable enough to be transported to other laboratories.

All drug standards received from the USAMRDC were logged into our record book and stored as required (protected against light, heat, or moisture). If necessary, they were checked for chemical purity or radio-purity by high pressure liquid chromatography or thin layer chromatography, purified through recrystallization or chromatography, and hydroscopic samples were dried according to USP methods.

Sample Generation for Assay Development

Spiked samples of biological media are generated by spiking different amounts of drug from known stock solutions into the biological media. Samples are mixed, then equilibrated for up to one hour at room temperature, unless the

compound of interest is unstable, in procedures in which it is especially important for measuring drug concentrations in blood, since drugs may take some time to reach equilibrium with erythrocytes.

Sample Preparation Procedures

Suitable preparation of the biological specimens is essential for the successful application of an analytical technique. The preparation procedure should be as simple as possible, yet allow for the specific measurement of the drug in the presence of numerous biological components. The extent of sample work-up is therefore largely determined by the selectivity and sensitivity of the analytical technique. Interfering endogenous substances must be removed before analysis. A second objective in devising preparation steps for a biological specimen is to protect the analytical apparatus from contamination by proteins and undissolved particles. Biological sample preparation thus varies according to the technical demands of the various analytical instruments utilized. Since the advent of highly selective analytical methods that combine chromatographic separation and detection in one unit [e.g. HPLC], the importance of the second objective has become more critical.

Protein Precipitation

Protein precipitation methods are rapid; they involve mixing the sample with water-miscible organic solvents. Acetonitrile yields a protein precipitate that can be readily centrifuged into a small pellet. Use of protein precipitation alone, without further work-up, is a popular application in HPLC analysis. It is possible, using appropriate measurement devices, such as electrochemical or fluorescence detectors, to obtain adequate sensitivity so that measurements in the nanogram per milliliter range can be made for drugs using small aliquots of the biological sample. We have used the protein precipitation method of sample preparation extensively in the development of analytical assays, including for antibiotics that are zwitterionic in nature, generally possess very low water-to-oil partition coefficients and, thus, are extremely difficult to extract efficiently. Also, protein precipitation is one method of choice for sample preparation, since a simpler sample preparation procedure reduces the risk of degradation. We use the direct protein precipitation method for our studies whenever possible (as demonstrated in Study Report 6 for mefloquine, Study Report 11 for WR 2721 and Study Report 12 for WR 3689).

Lower limits of quantitation with ultraviolet (UV) detectors are usually at about 50 ng/ml concentrations when the protein precipitation method is used. If UV detection is required, organic solvent extraction and solid phase extraction are more useful methods for preparation of biological samples for subsequent analysis. Extraction also limits column overloading and removes assay interferences.

Solvent Extraction

Three major variables were considered in the design of suitable organic solvent extraction procedures: the polarity of the organic solvent, the pH of the

aqueous phase, and the volumes of the organic and aqueous phases (as demonstrated in Study Reports 8 and 9 for physostigmine and its metabolite eseroline in plasma and Study Report 10 for WR 6026). A higher pH is often desirable since many endogenous substances are acidic and will not be extracted at alkaline pH. Consideration of pH is therefore important even when assays are developed for neutral drugs. Lipophilic bases are quite uncommon in body fluids, so it should be relatively easy to analyze many of the lipophilic basic drugs by extracting at high pH (as shown in Study Report 13 for WR 238,605 in plasma and blood and Study Report 14 for mefloquine). However, one solvent partitioning step alone is not always capable of separating bases from acids and neutral compounds. In such cases, multiple extraction steps must be employed.

A sample preparation method combining protein precipitation with acetonitrile and extraction with organic solvent is also a viable option. This method has been successfully used in our halofantrine assay ([2] and Study Report 17).

Commercial prepacked solid phase columns [e.g. Bond Elut™] with different types of packing materials, such as silica, C2, C8, C18 and ion exchange were employed. These columns are very useful for sample purification. Two approaches can be utilized: 1] the column separates desired compound(s) from interferences, or 2] the column retains desired compound(s), undesired endogenous substances are washed away, and the desired compound(s) are eluted with a suitable solvent. For low nanogram or picogram per milliliter concentrations, the method of retaining the desired compound on the column is preferred. This method has been successfully used in our laboratory for charged, water soluble compounds (pyridostigmine (see Study Report 5 for plasma and Study Report 7 for urine)), or highly nonpolar lipophilic, weakly basic and nonvolatile compounds (WR 6026[3] and halofantrine[2]) in biological fluids. For example WR 6026 and halofantrine are non-polar lipophilic compounds which are retained on C8 columns. Pyridostigmine, a quaternary amine, will not elute with CH_3CN alone. A 2 ml CH_3CN wash after loading the biological sample onto the C8 column eliminates undesired substances. The drug is subsequently eluted with CH_3CN containing SDS and tetramethylammonium chloride (TMA+Cl-) or 1% HCl culminating in a quantitation limit of 2 ng/ml with UV detection.

Specific functional groups in molecules of interest can also be advantageously used to purify biological samples by solid phase extraction. Diol functional groups can adsorb on a boronate column and subsequently be eluted with an acidic solution. This turned out to be our method of choice in the ribavirin and WR 249,992 assay development project (see Study Report 15).

Adsorption losses to glass or other apparatus for the low level lipophilic antimalarial drugs probably explains the inconsistent results reported by many investigators. The significance of this adsorption should be considered, especially when several extraction steps are to be employed. This was demonstrated during our development of the assay for halofantrine (WR 171,669) and its active metabolite, WR 178,460, in which WR 194,965 was used as

the internal standard ([2] and Study Report 17). The compounds were adsorbed by the glassware after reconstitution of the extract with organic solvent. In our experience, a true measurement of drug was obtained with the addition of a small amount of surface active agent to the solvent system before delivery onto the HPLC column. Adsorption loss can also occur in the port of delivery.

Detector Selection

The detector is a device that supplies an output in response to the presence of the compound(s) of interest. It is connected to the outlet of the column to monitor the column effluent in real time. The detector can be the most sophisticated and one of the most expensive components of a chromatographic system. Optical detectors, which currently dominate the field for biological samples in HPLC, include UV-visible absorbance detectors and fluorescence detectors. Depending on the measured difference between incidental and transmitted light intensity, these instruments can detect down to 9 to 10 ng of sample if the direct precipitation method is used. Electrochemical (EC) detectors are also used for routine work due to their specificity and/or sensitivity.

UV-Visible Absorbance Detector

Since the analytical methods for this contract required the quantitation of nanogram per milliliter concentrations of drug in biological samples, samples assayed with the UV detector required an extensive extraction work-up. For example, the pyridostigmine plasma assay was capable of quantitating 2 ng/ml concentrations of pyridostigmine (free base) (see Study Report 5) with UV detection only because of the extensive extraction procedure.

Fluorescence Detector

Fluorescence detection is more selective than UV spectroscopy. However, more structural requirements must be met to produce a high fluorescence yield (Ø) and to allow measurement above a negligible background (i.e., better quantitation limits). Minimum detection limits for the fluorescence detector can extend below the nanogram per milliliter level for favorable samples. (See Study Reports 9, 13, and 17).

Fluorescence intensity can be manipulated both by changes in solvent components and the pH of the solvent system. For example, quinoline is non-fluorescent in hexane but fluoresces in ethanol, while indomethacin shows fluorescence at a pH above 12. Most of the synthetic antimalarial drugs are asymmetrically conjugated, not strongly ionic and, hence, would be expected to fluoresce. Fluorescence detection might therefore be expected to be the method of choice for measuring antimalarial drugs due to the sensitivity, selectivity and lower dependence on instrumental stability (from pressure and temperature changes) of the detector.

Two different light sources at various wavelengths are used in commercial fluorescence detectors. They are the deuterium and the xenon arc lamps. The

xenon arc lamp has high intensity and the energy is more evenly distributed at different wavelengths, whereas the deuterium lamp emits at lower energy than the xenon arc and the intensity is drastically diminished at wavelengths above 280 nm.

Since the intensity of emitted fluorescence is dependent upon the intensity of the excitation source, it would appear that the sensitivity of a fluorescence assay can be increased without limit by using the most intense source. Many researchers do not realize that marked differences can be found with different lamp sources in commercial detectors.

8-Amino-quinoline antimalarial drugs, such as WR 6026, WR 238,605 and mefloquine (Study Reports 6, 10, 13, 14, 18, and 19) are highly conjugated and the excitation wavelengths were expected to be high. The xenon arc source equipped with monochronometers to collect both the excitation and emitted energy wavelengths provided us with maximum flexibility in fluorescence detection. With these devices, specific wavelengths for optimum sensitivity and/or selectivity were conveniently selected.

Electrochemical Detector

Electrochemical detectors (EC) are also used in methods of choice for applying liquid chromatography to trace (sub-nanogram) analysis. EC detection can provide the sensitivity and selectivity necessary for practical analytical procedures in a variety of situations. Material eluted from the chromatographic column acts at an electrode surface under controlled potential conditions and the current, which results from the net exchange of electrons, is monitored as a function of time. Since the amount of material converted by the electrochemical reaction is proportional to the instantaneous concentration, the current will be directly related to the amount of compound eluted as a function of time. The flow through a thin layer electrochemical cell is ideally suited for LC analysis since it can be easily constructed with a very small dead volume (1 µl) and maintain extreme sensitivity toward electroactive compounds. Several configurations using glassy carbon, carbon paste, or mercury-gold electrodes have been developed. If chromatographic conditions are carefully controlled, EC detection is quite precise and quantitative data can be obtained at the picomole level (total injected amount) for many compounds. In addition to being extremely sensitive, the electrochemical detector is quite specific in that only compounds electroactive at a given potential are detected. A large number of extremely important endogenous compounds, drugs, drug metabolites, food additives and organic pollutants are electroactive and therefore can be studied by EC. It is the method of choice for the detection of catecholamines and their analogs; numerous assay methods using EC detection have been published in the recent literature. We have been successful in using this detector for measuring the morphine analog, nalbuphine in urine. When determining whether or not a particular compound can be successfully analyzed by EC, it is not sufficient to know that the compound can react electrochemically. The type of electrode surface, the nature of the solvent system and relative ease of oxidation or reduction must be carefully considered before one can ascertain whether such an

analysis is feasible (see Study Reports 11 and 12 for phosphorothioate assays). Many important compounds have been studied in detail and conditions for analysis have been optimized. In order to assess fully the possibility of developing a new assay, it is desirable to carry out voltametric studies. This is equivalent to measuring an adsorption spectrum prior to using a UV detector.

With detection in the reductive mode, analysis of blood for artesunic acid and dihydroquinghaosu had been successfully carried out in Walter Reed Army Institute of Research.[4]

Phosphorothioates ($R\text{-}SPO_3H_2$) are potential radioprotective drugs investigated by the US Army. Neither UV nor fluorescence detection is suitable for this type of compound unless some other functional group in these molecules can be derivatized. To make matters worse, phosphoro-thioates are readily hydrolyzed to free sulfhydryl compounds in vivo (metabolism) and in vitro (degradation) and possibly further oxidized to disulfides. However, phosphorothioates can be detected by EC with dual mercury/gold electrode detectors connected in series. These can be very useful for the simultaneous determination of thiols and disulfides. Two Hg/Au electrodes are utilized in a series arrangement with reduction of disulfide to thiol at the upstream electrode, followed by conventional thiol detection downstream. The upstream electrode behaves as a novel on-line post column reactor of negligible dead volume. Phosphorothioates, thiols and disulfides are all readily quantitated in this detector and suitable separation is achieved by the HPLC system. It is interesting to recall that disulfide is actually being detected as the corresponding free thiol. No confusion occurs in measurements, however, because thiols are chromatographically resolved from the disulfide and thus separately detected.

LC/MS/MS

Mass spectrometric detectors are increasingly used in methods of choice for applying liquid chromatography to trace (sub-nanogram) analysis. Our Liquid Chromatographic/Mass Spectrometric/Mass Spectrometric (LC/MS/MS) systems for analysis of biological specimens require development and validation of procedures with PE Sciex-API III®, API 3000, Micromass Quattro LC Digital P-2 266I, or Ultima LC systems that use short liquid chromatography columns (3 or 5 μm particle size, 4.6 X 50 mm), the usual liquid chromatographic mobile phases, and mass spectrometric detection with sample inlet by heated nebulizer, positive ionization by APCI (Atmospheric Pressure Chemical Ionization) or electrospray positive or negative ionization and mass scanning by MRM (Multiple Reaction Monitoring) analysis.

Solvent System and Column

One of the most important steps in the development of an HPLC assay is selection of a suitable solvent system (mobile phase) and stationary phase. They are both closely related for maximum separation. Practical approaches are discussed in this section.

Reverse-Phase and Bonded Phase Columns

We intended to use reverse-phase systems for the majority of the analytical methods developed for HPLC assay described in this contract, since such bonded phase columns have several advantages for applications involving biological fluids. Reverse phase columns are stable since the stationary phase is chemically bonded to the support and cannot easily be removed or lost during use. Therefore, a pre-column and/or pre-saturation of the two phases is/are not required. Reverse-phase columns have minimal irreversible retention which is compatible with a large variety of solvents; it is often possible to inject an aqueous sample without further treatment. As a result, bonded phase columns (BPC) are especially suited for samples containing components with widely varying K' (column capacity factor). The availability of a wide variety of functional groups on BPC packing allows reverse phase and ion paired chromatography to be carried out in a relatively simple, straight-forward manner.

In reverse-phase liquid chromatography, water is the polar solvent and any less polar, water-miscible solvent can be used in conjunction. Common examples of the second solvent include methanol, acetonitrile and tetra-hydrofuran. The design of a successful LC separation depends on matching the right mobile phase to a given column and sample ion pairing mode.

Aqueous Mobile Phase and Silica Columns

The recent use of an unbonded silica stationary phase and an aqueous mobile phase has been successfully used in our laboratory for the liquid chromatographic separation of lipophilic amines. When C18 bonded phase columns are used, it is often necessary to employ amine mobile phase modifiers to ensure good retention times and peak shapes in the ion-suppression mode. Recent work suggests that unbonded silica gel, with the maximum concentration of surface silanol groups, is a preferable stationary phase for these compounds. Use of unbonded silica as the stationary phase permits the separation of a wide variety of amine compounds with a simple mobile phase containing an organic solvent and an aqueous phosphate buffer at neutral to alkaline pH. The retention volumes are lower and the peaks are more symmetrical when silica, rather than a C18 bonded support, is used as the stationary phase. The method is especially suitable for assays of biological fluids, since endogenous non-ionic neutral lipid compounds and anionic compounds will not be retained on the silica gel column while cationic aliphatic amine drugs will be retained. The interfering substances in biological fluids are eluted at the solvent front, leaving a very clean base line about the drug's retention time. Using a silica column and an aqueous solvent system, we obtained a quantitation limit of 2 ng/ml for plasma samples for pyridostigmine (free base) (see Study Report 05).

Selectivity and Resolution Modification

As a general approach to increasing (column selectivity) and improving resolution, several options are available and can be ranked in order of decreasing promise.

Modification of Mobile Phase

Many different properties of the solvent must be considered, including solvent strength and selectivity. Polar compounds are best separated by a polar solvent system, while non-polar compounds should be separated with a less polar system. Separation may be defined as the ability of the solvent system and column material to retain the compound of interest on the column for a longer period of time than the undesired components. We found that a change from methanol to acetonitrile can sometimes enhance the selectivity of the column. We tend to use acetonitrile as the solvent modifier since it has a lower viscosity and tends to increase the efficiency of the column; it is also characterized by increased miscibility with non-polar samples.

Change of pH and Ionic Strength

Aqueous buffers are commonly employed to suppress ionization of the ionizable sample components in reverse-phase analyses. The pH of the mobile phase is varied and the resulting changes in K' (column capacity) and alpha (column selectivity) are examined.

TABLE 3: DRUGS IN PLASMA ASSAYED WITH A SILICA GEL COLUMN AND AN AQUEOUS MOBILE PHASE

Drug	Detection Limit (ng/ml)	Detection Mode	Sample Preparation	Mobile Phase	Retention Time (min.)
WR 238605	0.8	Fluorescence	Liquid extraction	50% CH_3CN 5 mM $(NH_4)_2HPO_4$ pH = 7.0	6.50
WR 6026	1.0	UV	Liquid extraction	60% CH_3CN 5 mM $(NH_4)_2HPO_4$ pH = 7.0	7.00
Halofantrine	1.0	Fluorescence	Liquid extraction	80% CH_3OH 5 mM $(NH_4)_2HPO_4$ pH = 8.2	7.00
Pyrido-stigmine	1.4	UV	Solid Phase extraction	50% CH_3CN, 0.05% TMAC 5 mM $(NH_4)_2HPO_4$ pH = 7.2	16.4
Mefloquine	8.0	UV	Liquid extraction	80% CH_3OH 5 mM $(NH_4)_2HPO_4$ pH = 7.5	10.1

Strength of buffers or ion-pairing agents can also influence the retention times of many drugs. Most of the antimalarial drugs are highly hydrophobic in nature, hence a high ratio of organic solvent modifier should be required and ion-pair techniques will be involved.

For silica stationary and aqueous mobile phase systems, the interaction between silica and amine is electrostatic and the separation mechanism is similar to an ion-exchange mechanism. Here, the pH of the mobile phase (pH 7-9.5) and pK_a of the amine are very important in determining retention time, while the pH of the mobile phase in bonded phase systems (pH 2-5) is not as critical. Ionic strength is also critical. Thus for silica gel-aqueous mobile phase systems, mobile phase pH and ionic strength are more important to retention time determination than the organic modifier (e.g. CH_3CN, CH_3OH), which is the critical determinant in bonded gel - reverse-phase systems.

Change of Stationary Phase

A change of stationary phase is less convenient than a change in mobile phase composition and is less commonly used. Further adjustment of the mobile phase composition is usually required when a new column packing or stationary phase is used to optimize of solvent strength and K' values.

Most aromatic antimalarial drugs are very non-polar. It is reasonable to expect that the retention time will be shorter on a more polar C8 column (tend not to retain) than a non-polar C18 column. This was found to be true during the development of our first WR 171,669 (halofantrine) assay. The retention time for this compound is reduced by 1/3 by changing from a C18 column to a C8 column when the same mobile phase is used.

Temperature Change

The fourth technique for varying K' values is to increase (or decrease) the temperature. Since an increase in temperature normally reduces all sample K' values, it is usually necessary to decrease solvent strength to compensate for this effect. A change in temperature usually has little effect on sample K' values in liquid-liquid chromatography, but it is important in ion exchange and ion-pair chromatography. For this reason, a change in temperature for improvement of K' in ion-exchange and ion-pair chromatography is generally more promising than a change in stationary phase.

Complexation

A final means of changing K' values, sometimes dramatically, is through chemical complexation. A well known example is the use of metal ions (e.g. $Ag^+NO_3^-$) in the solvent system to separate various olefinic compounds. The complexation of olefin and metal ion causes dramatic changes in retention time and selectivity.[5] This technique is probably applicable for some antimalarial drugs.

For the most part, we intended to use C8 columns and/or ion-pair techniques to develop assay methodologies. However, silica gel column - aqueous mobile phase systems are our general method of choice for amines. Since measurement concentrations of 5 to 20 ng/ml are required, we expected to use 5 μm particle size columns for separation of drugs.

Derivatization

Derivatization is an important adjunct to HPLC assays. The choice of derivatization procedure is dependent upon the type of detector that is used. We are actively involved in pre-column and post-column derivatization as well as in structure modification studies to increase detection sensitivity. A wealth of information on potentially useful derivatives is available from the disciplines of qualitative organic analysis[6] and protective group synthesis.[7] In choosing a derivative for HPLC, ideally the reaction should be specific, quantitative, free from side reactions, complete in a relatively short time, and done under mild conditions. This kind of information is not readily available in the literature, and therefore, derivatization studies can be a rather time consuming venture. The design or choice of a derivatizing agent is critical.

Post column derivatization or degradation is also an excellent way to increase sensitivity of an assay. The technique of post column hydrolysis at alkaline pH and post column oxidation reactions with potassium permanganate or potassium periodate can be applied to assays that employ fluorescence detection. Post-column photo-irradiation is another way to increase sensitivity. First, the drug of interest is separated from other components of the sample by HPLC. Then, the sensitivity is enhanced by photo-irradiation, which may rearrange the chromophore or otherwise break bonds to form a fluorescent species

Assay Validation

Validation of each method was performed using biological fluid obtained from same species, when possible. This process indicates sample stability, method precision, accuracy and selectivity, and the feasible sample concentration range for use in pharmacokinetic or bioavailability studies. Validation procedures are part of our standard operating procedures (SOP) which are written in accordance with our program to meet Good Laboratory Practice (GLP) regulations. The procedures are described in the Analytical Section Procedural Manual, Procedure 2D-3.10 "Procedure for Validation of an Assay Methodology" and earlier versions. The following sections summarize Procedure 2D-3.10.

Specificity

The specificity should be evidenced by showing with chromatograms that: Test compounds are separated from major metabolites (if metabolite standard is available); Test compounds are separated from co-administered drugs (if any); At least six different sources of biological fluid should be free of possible interference by endogenous peaks at the retention times of the test compounds and internal standard. Biological fluid means blood, plasma, and urine. For rare

biological fluids, such as aqueous humor, bile juice, feces, bone cartilage, etc., two sources are tested, if available. These chromatograms are compared to a lower limit of quantitation chromatogram.

All assay methods developed required use of an internal standard. Analogs of the compounds under study or chemicals with similar functional groups were preferred as internal standards. The internal standard must elute at a different time than the drug of interest, yet separate from endogenous substances in the biological sample. In addition, it should have similar extraction or partition properties as the drug of interest during the sample preparation process.

Linearity

A calibration curve is generated from a blank (00), zero (0) and eight or more standard calibrator samples. Linearity is demonstrated by acceptable spiked vs. calculated concentrations (or vs. peak response ratios), y-intercept, and coefficient of determination (r^2) values for the standard curve.

Calibration curves were constructed from the peak height (or peak area) ratio of drug to internal standard versus spiked concentration of drug by linear regression (unweighted or weighted method).

In the weighted least squares linear regression method, weights (w) = $1/y_i$, the intercept, *b*, is defined by:

$$b = \frac{\left(\left(\sum w_i x_i^2\right)\left(\sum w_i y_i\right)\right) - \left(\left(\sum w_i x_i\right)\left(\sum w_i x_i y_i\right)\right)}{\left(\left(\sum w_i\right)\left(\sum w_i x_i^2\right)\right) - \left(\sum w_i x_i\right)^2}$$

and the slope, *m*, is defined by:

$$m = \frac{\left(\left(\sum w_i\right)\left(\sum w_i x_i y_i\right)\right) - \left(\left(\sum w_i x_i\right)\left(\sum w_i y_i\right)\right)}{\left(\left(\sum w_i\right)\left(\sum w_i x_i^2\right)\right) - \left(\sum w_i x_i\right)^2}$$

Two standard curves may be calculated from the same set of standard curve calibrators (unless the weighted linear regression method is used). The low range curve is calculated from low concentration standard curve points and is used to derive concentrations from samples with peak response ratios at or below the calculated peak response ratio of the highest standard curve point used in the low range curve. The high range curve is calculated from all standard curve points and is used to derive concentrations from samples with peak response ratios above the calculated peak response ratio of the highest standard curve point used in the low range curve.

Standard curve results are reported in a table containing spiked concentrations, peak response ratios, calculated concentrations, slope(s), intercept(s) and r^2 value(s) of a typical standard curve that was used in the

method validation and in a table containing all slopes, intercepts and r^2 values of standard curves run in "intraday" and "interday" studies.

Lower Limit of Quantitation

The lower limit of quantitation is defined as the lowest standard curve concentration which can be reasonably, accurately, and precisely quantitated. The analyte response at the LLOQ should be at least 5 times the response in the blank sample. Six samples spiked to the lowest standard curve concentration and a standard curve are prepared. The samples are run together within one day (or one run). The 6 lowest point of the standard curve sample concentrations, and their mean, S.D., C.V. (percent) and deviation (percent) are calculated. These data are used as the quantitation limit intraday result.

The 6 calculated lowest point of the standard curve concentrations that were obtained in the interday precision study and their means, S.D.s, C.V. percents and deviation percents are used as the quantitation limit interday result.

Recovery

It is important to check the recovery of compounds of interest during the assay in order to assess the uniformity of recovery during the assay or whether or not a better recovery can be obtained. Radio-labeled drugs, when necessary, were added to the sample and either the direct precipitation, solid phase purification or the extraction procedure was utilized to evaluate recovery. If labeled compounds were not available, a recovery study similar to those for WR 6026,[3] halofantrine and its metabolite, WR 178,460,[2] and pyridostigmine[8] were carried out. In brief, the recoveries of these drugs from plasma or whole blood were determined by comparison of the drug-to-internal standard peak height ratios of blood or plasma versus water samples spiked with the drug. In each case, the internal standard was added after sample was eluted from the solid phase column, extraction from organic solvent, or direct precipitation with CH_3CN to insure that the internal standard did not bind to the blood or plasma or to the cartridge during the preparation.

Recovery of the internal standard (IS) (at the IS concentration used in the study) is tested against the drug (at a drug concentration corresponding to that of the medium control). Recovery is assessed as the ratio of the IS's peak response ratios (IS to drug) in an extracted sample to an unextracted sample for triplicate spiked samples.

Precision

Precision is expressed as the standard deviation (S) of the assayed concentration where Xi are the repeated concentration measurements of an individual sample and $\overline{x}$ is the mean concentration.

$$S = \left(\frac{\sum_{i=1}^{N} (Xi - \overline{x})2}{(N - 1)} \right)^{\frac{1}{2}}$$

The coefficient of variation (C.V.) was used for determination of the precision. The sample number was 6 for intraday and 12 for interday precision.

If needed, the assay results were compared to those obtained with an assay of proven reliability and specificity. For example, the Pearson correlation coefficient (r) can be used. Maximum r value indicates exact correlation between the two variables and r = 0 indicates complete independence.

$$r = \frac{(Si - \overline{s})(Yi - \overline{y})}{n \bullet Sx \bullet Sy}$$

The within-run precision was determined by measuring the concentration of drug in six biological samples for each of three sets of low, intermediate and high drug concentrations. The results for each set are used to calculate the standard deviation and coefficient of variation (CV). The between-run precision is measured on separate days with six sets of duplicate biological samples at low, intermediate and high drug concentrations. From these samples, the between-run standard deviation and CV are calculated for each drug concentration.

Accuracy

Accuracy was determined by assaying a series of blind samples prepared according to the project director of DAMD. Estimates of the accuracy of the method over the standard curve working range were also determined in the precision analysis by the analysis of replicate spiked samples for intraday (n = 6) and interday (n = 12) precision. Results were expressed as relative error (RE) with respect to the spiked concentration.

Matrix effect

Blank plasma or other biological fluid (samples spiked with drug(s), (metabolites,) and internal standard after clean-up treatment, i.e. precipitation, extraction-reconstitution, elution) are tested from at least 6 different sources versus extraction solvent/mobile phase samples (appropriately treated and correspondingly spiked) in duplicate at the M concentration for drug(s) (and metabolites) and at about the method concentration for the internal standard to show any matrix effect. Peak area data for drug(s), (metabolites,) and internal standard are reported.

Multiple drug method calibration curves (cross talk effect)

In multiple drug methods, one standard curve for each drug alone in biological matrix as well as for a mixture of all drugs is generated. For each

drug, absence of any peaks at the corresponding retention times of the other drugs is determined.

Anticoagulant Interference (plasma studies only)

At least three sets in duplicate are generated of blank and M concentration (spiked samples with drug(s), (metabolites,) and internal standard after clean-up treatment, i.e. precipitation, extraction-reconstitution, elution) plasma samples, each set containing a different anticoagulant (i.e. EDTA, CPD or CPDA, and Heparin). These samples are run together within one run to determine whether anticoagulant interference occurs.

Re-injection

If the method processed sample volume is adequate, re-injection is tested by twice re-injecting an interday precision run. Results of the individual concentrations of three control samples at each concentration level and their means (n=2), CV% for precision, and relative error for accuracy are used to determine whether re-injections can be performed.

Dilution

A sample to be diluted that is 5 times the concentration of the H control is generated. Three aliquots, each diluted 5 times are assayed. Results of the individual concentrations of the three diluted samples, their means (n=3), CV% for precision, and relative error for accuracy are used to determine whether sample dilutions can be performed.

Stability

Stability studies of a drug in biological media serve to establish the procedure for proper storage of the samples and furnishes information to clinical researchers on how best to handle these occasionally labile samples. We have a great deal of experience in planning and executing the required stability studies. In methods developed for analytical and clinical studies, drug stability may play a particularly important role.

Known amounts of sample in different biological media are measured at various times after preparation and assayed for the drug, in duplicate. Variables, including light exposure, storage conditions (container type) and pH of the biological samples, are evaluated if necessary.

Since clinical samples are often repeatedly assayed and samples are thawed and refrozen, it is necessary to check for any instability of samples during these processes. Practically, this study can be done by using two concentration samples (High and Low). The same volume of biological fluid used to prepare standard curve samples is aliquoted to the appropriate number of tubes. Samples (in duplicate) are thawed and refrozen (a cycle) for 5 cycles. Samples are repeatedly thawed and refrozen according to the following table. Samples

are thawed as if for sample preparation to room temperature and are left to stand at room temperature for 1 hour.

Cycle	Keep these samples in freezer
1	a
2	a, b
3	a, b, c
4	a, b, c, d
5	a, b, c, d, e

Following Cycle 5, all of the samples are thawed to room temperature and assayed with a standard curve. Test sample concentrations are calculated and reported in a table for each concentration (n=2) of mean concentrations (n=2) at each test point (n=5).

System (processed sample) stability: Stability of drug and internal standard in biological samples prepared for analysis as described above under "Sample Preparation" were demonstrated by assay of sets of control samples with concentrations to cover the standard curve range. Assay results were obtained for prepared samples that were left standing at room temperature for various times after preparation.

Refrigerated (processed sample, refrigerated) stability: Assay results were obtained for prepared samples that were left standing at 4°C for various times after preparation.

Long term stability: Stability of a drug in a biological specimen stored at -20°C and/or -70°C was demonstrated by assay of sets of control samples prepared as described under "Sample Preparation" at concentrations to cover the standard curve range. Long term stability samples were kept at -70°C or -20°C until prepared and analyzed.

Bench top (unprocessed sample) stability: Plasma samples were left to stand at room temperature for various times after generation, then were kept at -20°C and/or -70°C until prepared and analyzed.

Stability of standard and internal standard solutions is evaluated at room temperature, if necessary, and at solution storage temperature to cover the period of use.

Purity of Standard Chemicals

The standard chemicals used in a study are USP™ reference standards (if available) or pure chemicals provided by the sponsor, unless otherwise instructed.

A chemical purchased from a general chemical company is not used as a standard, except in unusual circumstances and when its purity can be verified against a USP™ reference standard or the sponsor's standard verified by a certificate of analysis. The internal standard is not under this restriction.

USP™ reference standards can be regarded as 100% pure (unless specified), and no purity correction factor for concentration calculations is necessary. However, a sponsor's standard chemical must be regarded as possibly impure and a correction factor should be considered.

When verifying non USP™ reference standard chemical purity, the working standard solution is run under the method used for assaying biological samples. Each solution is injected 3 times, and two standard solutions are prepared for each standard chemical.

A copy of the supplier's certification of analysis (COA) sheet is saved with the method validation files.

Routine Assay Procedure

The following are the steps carried out when samples arrive for routine analysis. Sample arrival is recorded in the sample log-in book and on the log-in sheet, which includes the name of the shipper, arrival date, number of samples, sample storage location, and sample condition. An analytical procedure (AP) is normally completed prior to routine analysis. In this AP, the method description is condensed to about 3-10 pages that contain information regarding instrumentation, assay conditions, source of chemicals, preparation of stock solutions, sample preparation and representative chromatograms.

For routine sample analysis, standard curve, blank and control samples are also analyzed. Sets of equipment consisting of a pump, detector, column, integrator and autosampler or the LC/MS/MS system were set up for routine assay. Each system is tested by assigning personnel to run a series of controls; the performance of equipment and technical personnel are validated before routine sample assay.

Carry over testing is performed for each run by assay of a blank sample (not spiked with internal standard or with drug standard) immediately following the assay of a high concentration control.

To monitor variation during the course of assay of a sufficient quantity of samples, a series of controls are prepared beforehand and stored in the freezer. Control samples are run together with standards and routine assay samples. Each set of controls normally includes three different concentrations within the range of the standard curve. For every group, treatment, or up to 20 routine assay samples, a set of controls (e.g. low, medium and high) is included before and after the set to validate the results. Since the concentrations of the controls are known, it is possible to judge whether the routine assay samples must be repeated on the basis of the results obtained for the controls.

Assay samples are prepared by spiking known volumes of biological sample with a known amount (constant over all samples) of internal standard (IS). Standard curve samples are generated by spiking interference free biological samples with known amounts of standard compound and IS. These standard curve and assay samples are prepared according to the analytical procedure, then injected onto an LC column for separation and subsequent detection. The peak response ratio of standard compound to IS is calculated for each sample from the measured peak response obtained by HPLC or LC/MS/MS. Finally, spiked concentrations and standard compound to IS peak response ratios of the standard curve samples are fit by weighted or non weighted least squares linear regression to the equation for the best straight line ($y = mx + b$, where y = peak response ratio and x = standard compound concentration), and standard compound concentrations in assay samples are calculated by this equation from the standard compound to IS peak response ratios obtained by HPLC or LC/MS/MS.

Assay findings are then sent in a report with a complete assay methodology including detailed methods, statistical evaluation of methods, routine assay sample results, results from the control samples, and one representative set of calibration chromatograms. Results can be sent by disc or through a modem for pharmacokinetic evaluation.

Experimental methods

The goals of the research under contract DAMD17-97-C-7058 are 1) to develop and validate methods to assay for drug substances in biological fluids for pharmacokinetic, bioavailability, drug metabolism and drug monitoring studies, and 2) to use these methods to perform routine analyses of biological specimens to support pharmacokinetic and bioavailability studies as part of preclinical and clinical investigations undertaken for the purpose of new drug development.

Method Development and/or Validation Results

The following section describes the status of specific methods developed and validated or currently being developed and/or validated during the contract. Completed validation methodologies are presented in Appendix A, except those presented in earlier annual reports. Table 4 presents current validation studies, and Table 5 presents current routine analysis studies.

TABLE 4: CURRENT VALIDATION STUDIES

Report No.	Report Date	Report Title	Test System	Test Article	Lower Limit of Quantitation
21	Draft report in preparation	Tentative title: Quantitation of *p*-Aminoheptanophenone, *p*-Aminooctanophenone, and *p*-Aminopropiophenone in Dog Plasma by HPLC	dog plasma	PAHP PAPP PAOP	4.08 ng/ml 4.04 ng/ml 4.16 ng/ml
22	Draft 7/18/94 in revision	Quantitation of WR 6026, WR 211,789, and WR 254,421 (as Free Bases) in Human Urine By HPLC	human urine	WR 6026 WR 211,789 WR 254,421	5.17 ng/ml 5.09 ng/ml 45.4 ng/ml
28	2/9/01 draft report in review	Short Validation of a HPLC Method for the Determination of R & S Isomers of Halofantrine and WR 178460 in Human Plasma Samples	human plasma	Halofantrine WR 178,460	ng/ml ng/ml
29A	August 11, 2000 final report	Validation of a LC/MS/MS Method for the Determination of Chloroquine and Monodesethylchloroquine in Human Blood Samples	human blood	Chloroquine (C) MonodesethylC	20 ng/ml 20 ng/ml
29B	9/23/99 draft report in review	Validation of a LC/MS/MS Method for the Determination of Chloroquine and Monodes-ethylchloroquine & Dides-ethylchloroquine in Human Blood & PlasmaSamples	human blood human plasma	Chloroquine (C) MonodesethylC DidesethylC Chloroquine (C) MonodesethylC DidesethylC	20 ng/ml 20 ng/ml 20 ng/ml 20 ng/ml 20 ng/ml 20 ng/ml
30	In validation	Tentative title: Quantitation of WR 243251 in Human Plasma by LC/MS/MS	human plasma	WR 243251	1 to 5 ng/ml
32	Draft in preparation	Tentative title: Quantitation of WR 238,605 in Human Plasma and Blood by LC/MS/MS and	human plasma blood	WR 238605 WR 238605	ng/ml ng/ml

TABLE 4: CURRENT VALIDATION STUDIES

Report No.	Report Date	Report Title	Test System	Test Article	Lower Limit of Quantitation
32, S1	April 12, 2000 draft report in review	Supplement I: Validation of a LC/MS/MS) Method for the Determination of WR 238605 in Small Volume Human Blood Samples	human blood	WR 238605	5.00 ng/ml
33	In validation	Tentative title: Quantitation of Halofantrine and WR 178,460 in Human Plasma by LC/MS/MS	human plasma	Halofantrine WR 178,460	ng/ml ng/ml
34	In development	Tentative title: Quantitation of WR 254421 in Human Plasma by LC/MS/MS	human plasma	WR 254421	ng/ml
35 S1	May 25, 2001, draft in review	Validation of a LC/MS/MS Method for the Determination of Artelinic Acid in Human Plasma Samples, Supplement I	human plasma	Artelinic Acid	4 ng/ml
35 S2	In validation	Tentative title: LC/MS/MS Method for the Determination of Artelinic Acid in Rat Plasma Samples	rat plasma	Artelinic Acid	4 ng/ml
35 S3	In developmen	Tentative title: LC/MS/MS Method for the Determination of Artelinic Acid in Monkey Plasma Samples	monkey plasma	Artelinic Acid	4 ng/ml
35 S4	In developmen	Tentative title: LC/MS/MS Method for the Determination of Artelinic Acid Metabolites and Artesunate in Rat, Dog and Human Plasma Samples	dog human rat plasma	artesunate metabolite 1 metabolite 2	ng/ml
36	In development	Quantitation of Paromomycin and Gentamicin in Human Urine by HPLC	human urine	Gentamicin Paromomycin	ng/ml

Study Report 18: WR 6026 and Metabolite in Human Plasma and Blood

Study Characteristics: Study Report 18

Test Article:	WR 6026, WR 211,789
Test System:	human plasma and blood
Internal Standard:	chlorpheniramine
Sample Assay Volume:	0.5 ml
Sample Cleanup:	methyl *t*-butyl ether extraction

Analytical System

Detector:	UV at 263 nm
Column Type:	silica
Column Size:	4.6x250 mm, 5μ particle size
Mobile Phase:	acetonitrile/water (3:2, v/v) final concentration of 5 mM $(NH_4)_2HPO_4$ at pH 8.8

Validation Results: WR 6026 in human plasma

Quantitation Limit:	0.980 ng/ml
Standard curve range:	0.980-98.0 ng/ml
Interday Precision	
Concentration Range:	2.06-77.3 ng/ml
CV Range:	3.05-6.82%
Intraday Precision	
Concentration Range:	2.06-77.3 ng/ml
CV Range:	3.22-9.39%
Blind Sample Assay	see Appendix A, DAMD17-92-C-2028 Midterm Report
Mean Recovery:	74.5%
Stable Plasma Storage:	-20°C for 3 months

Validation Results: WR 211,789 in human plasma

Quantitation Limit:	1.21 ng/ml
Standard curve range:	1.21-121 ng/ml
Interday Precision	
Concentration Range:	2.14-80.1 ng/ml
CV Range:	5.19-8.98%
Intraday Precision	
Concentration Range:	2.14-80.1 ng/ml
CV Range:	4.42-7.86%
Blind Sample Assay	see Appendix A, DAMD17-92-C-2028 Midterm Report
Mean Recovery:	93.8%

Validation Results: WR 6026 in human blood

Quantitation Limit:	0.980 ng/ml
Standard curve range:	0.980-98.0 ng/ml
Interday Precision	
Concentration Range:	1.96-78.4 ng/ml
CV Range:	1.56-6.38%
Intraday Precision	
Concentration Range:	1.96-78.4 ng/ml
CV Range:	2.31-5.36%
Stable Plasma Storage:	-20°C for 1 month -70°C for 3 months

Validation Results: WR 211,789 in human blood

Quantitation Limit:	1.21 ng/ml
Standard curve range:	1.21-121 ng/ml
Interday Precision	
Concentration Range:	2.40-96.0 ng/ml
CV Range:	1.74-5.12%
Intraday Precision	
Concentration Range:	2.40-96.0 ng/ml
CV Range:	1.76-4.85%

Study Description: WR 6026 and Metabolite in Human Plasma and Blood (the methodology was presented in DAMD17-92-C-2028 mid-term report)

Sets of blind plasma and blood samples, prepared April 1, 1993, were received. Blind plasma sample results were enclosed with Quarterly Report 8. Upon analysis of blood samples, results will be forwarded to the COR. Acceptable results will be incorporated into Study Report 18, "Quantitation of WR 6026 and WR 211,789 (as Free Bases) in Plasma and Blood by High-Performance Liquid Chromatography." The test of stability is in progress.
Procedures Required to Complete Validation

The following list details changes that were instituted for plasma sample analysis, but that have not been tested for validation of the blood sample analytical method.

1. Following addition of 5 ml of methyl-*t*-butyl ether, vortex [not rotate] samples for 1 [not 15] min.
2. Adjust the mobile phase pH to 8.8 [not 7.0].
3. Stock and working solutions were stored at -20°C [not 4°C].

The following list details validation tests that have not been done.

1. Stability of WR 211,789 (free base) at -80°C and -20°C in blood and plasma.
2. Recovery of WR 6026 and WR 211,789 from blood.

3. Precision of WR 6026 and WR 211,789 (as free bases) in blood with mobile phase pH = 8.8, storage of stock and working solutions at -20°C, and vortexing extraction samples for 1 min.
4. Accuracy for WR 6026 and WR 211,789 (as free bases) in plasma and blood on blind spiked samples prepared by the Walter Reed Army Institute of Research.
5. Interference: To determine whether known compounds would interfere with detection of WR 6026 or WR 211,789 (as free bases), the retention times relative to CPA in mobile phase of several WR 6026 (free base) analogs could include WR 225,742 and WR 254,421 (free base).

Study Description

WR 6026 (dihydrochloride) (6-methoxy-8-(6-diethyl amino hexyl amino) lepidine dihydrochloride) (see figure below), is a very effective antileishmanial drug in hamsters infected with *Leishmania donovani* .[9]

Because antimony compounds are not always effective and the other drugs in use have toxic effects,[10,11] alternative therapies are needed. Since WR 6026 (dihydrochloride) is a likely candidate and since WR 6026 (dihydro-chloride) is scheduled for clinical testing in the near future, it is extremely important to develop an analytical method capable of measuring concentra-tions of WR 6026 (free base) at nanogram per milliliter concentrations in biological samples.

This report describes an assay developed to determine the concentrations of WR 6026 and of its mono dealkylated metabolite, WR 211,789, (as free bases) in blood and plasma. This new assay provides significant improvements over capabilities of earlier assays with increased sensitivity for the detection of WR 6026 (free base)[12]and inclusion of WR 211,789 (free base) in the methodology (Study Report 10).

Plasma samples (0.5 ml transferred with a plastic tipped pipetter to silanized culture tubes (see SOP #3-11 for silanization procedure)) were vortexed with 100 µl of a 1.00 µg/ml chlorpheniramine maleate internal standard working solution and 100 µl of a 1 N NaOH solution for 10 s. Next, 5 ml of methyl-*t*-butyl ether was added and samples were vortexed for 1 min, then centrifuged for 10 min at 3000 *g*. Then, for each sample, the aqueous layer was frozen in a dry ice/methanol bath and the organic layer were decanted into a new silanized culture tube. Finally, the sample's organic layer was evaporated to dryness under prepurified nitrogen, reconstituted in 200 µl of mobile phase, vortexed for 1 min, transferred to silanized WISP inserts, and injected onto the HPLC column.

Blood samples (0.5 ml transferred with a plastic tipped pipetter to silanized culture tubes) were vortexed for 1 min with 0.5 ml of nanopure water, and the mixtures were sonicated for 10 min. Then, these samples were prepared like plasma samples beginning with addition of 100 µl of the internal standard working solution.

No degradation of WR 6026 (free base) in plasma frozen at -20°C or blood frozen at -80°C was seen for the duration of the stability study. However, noticeable degradation of WR 6026 (free base) in blood frozen at -20°C was observed by the third month at all concentrations.

Two standard curves for each assay were constructed from the chromatographic data; a low range curve from the 0 to 14.7 ng/ml for WR 6026 and 0 to 18.1 ng/ml for WR 211,789 standard curve samples and a high range curve from the 0 to 98.0 ng/ml for WR 6026 and 0 to 121 ng/ml (i.e. all) standard curve samples in order to obtain more accurate determinations of low level WR 6026 and WR 211,789 (free base) concentrations. The low range standard curve was used to calculate drug or metabolite concentrations for assayed samples when the peak height ratio of the sample was less than or equal to the calculated peak height ratio at the highest concentration of the low range curve (as calculated from the low range curve). The high range curve was used to calculate results for samples with peak height ratios greater than the calculated peak height ratio at the highest concentration of the low range curve (as calculated from the low range curve).

Typical plasma and blood chromatograms show WR 6026 (free base), WR 211,789 (free base) and internal standard, chlorpheniramine, peaks that are baseline separated and separated from other components of the sample.

A linear relationships was demonstrated between the WR 6026 and WR 211,789 (free base) spiked concentrations to the WR 6026 and WR 211,789 (free base) to internal standard peak height ratios for the plasma and blood assays. Linear regression analysis of concentration versus the peak height ratio gave coefficients of determination (r^2) of 0.989 or better for these typical standard curves. The linear range of the standard curves covered WR 6026 (free base) concentrations in plasma and blood in the range 0.980 to 98.0 ng/ml and WR 211,789 (free base) concentrations in plasma and blood in the range 1.21 to 121 ng/ml. The reversed-phase system (alkyl bonded silica gel with an aqueous mobile phase) is the most widely used HPLC technique in assays for drugs in biological fluids. In this kind of a system, the retention mechanism depends mainly on the lipophilic character of substances to be analyzed. Such a mechanism also retains considerable amounts of other lipophilic substances, thereby interfering with the drug peak. On the other hand, in a system consisting of a bare silica gel and an aqueous mobile phase, the retention mechanism results mainly from ion exchange[13] and only partially from lipophilic interactions. Thus, endogenous non-ionic neutral lipid compounds and anionic compounds will not be retained on the silica gel column; only the cationic (e.g. ammonium) ions will be retained. The interfering substances in biological fluids elute at the solvent front, leaving a very clean baseline around the retention time of the drug.

Validation trials in our laboratory for an earlier study (Study Report 10) were undertaken to include in the WR 6026 (free base) assay the capability to measure WR 211,789 (free base), a mono dealkylated metabolite of WR 6026 (free base), concentrations in biological samples. Large variations between spiked and

recovered concentrations were observed in that study. Although WR 211,789 has been detected in a rat microsomal preparation,[14] it has not been detected in plasma in human studies, perhaps because the detection limit of the assay used was only 10 ng/ml.[19] WR 211,789 plasma standard curves in the trials were of higher quality than blood standard curves. The current report describes an adaptation of the WR 6026 (free base) methodology or a modification of the methodology presented in the earlier report (Study Report 10), in which a 5 to 10 fold increase in sensitivity has been gained that makes detection of WR 211,789 (free base) in human plasma possible at higher WR 6026 (dihydrochloride) doses.

In addition, compared to an even earlier methodology,[19] the WR 6026 (free base) HPLC method presented here offers increased sensitivity and extends the range of biological fluids that can be assayed. The earlier method measured WR 6026 (free base) in plasma cleaned by protein precipitation (with acetonitrile) and column elution (from a C2 extraction column), had a 6.44 ng/ml WR 6026 (free base) detection limit, used WR 223,658 as an internal standard, required a C8 bonded silica gel HPLC column, used a 60:40 (v/v) acetonitrile/water mobile phase at pH 5.5 with 0.2% final concentrations of SDS and glacial acetic acid, and measured WR 211,789 (free base) with a minimum detection limit of 8 ng/ml. The newer method measures WR 6026 (free base) in plasma and blood cleaned by extraction with 99:1 (v/v) pentane/acetonitrile, has a 0.980 ng/ml WR 6026 (free base) detection limit, uses chlorpheniramine maleate as an internal standard, requires an unbonded silica gel HPLC column, uses a 70:30 (v/v) acetonitrile/water mobile phase at pH 7.0 with 5 mM final concentration of dibasic ammonium phosphate, but could not measure WR 211,789 (free base) with a minimum detection limit much better than 8 ng/ml. The current modified method measures WR 6026 and WR 211,789 (free base) in plasma and blood cleaned by extraction with methyl-*t*-butyl ether, has 0.980 ng/ml WR 6026 and 1.21 ng/ml WR 211,789 (as free bases) detection limits, uses chlorpheniramine maleate as an internal standard, requires an unbonded silica gel HPLC column, uses a 60:40 (v/v) acetonitrile/water mobile phase at pH 8.8 with 5 mM final concentration of dibasic ammonium phosphate.

HPLC assays for basic amine drugs in biological samples that make use of a silica gel column and an aqueous mobile phase have been operated in this laboratory for over 5 years.[15,16,17] In the WR 6026 (free base) HPLC method presented here, the use of an unbonded silica gel column, an aqueous mobile phase, and UV detection at 263 nm yields satisfactory results for the determination of WR 6026 and WR 211,789 (as free bases) in (0.5 ml) plasma and blood samples. The method is simple in that a single extraction step and evaporation of solvent prior to injection are required. Recovery of WR 6026 (free base) averaged 74.5%, while recovery of WR 211,789 (free base) averaged 93.8% from plasma. The minimum quantitation limits of the assay were 0.980 ng/ml for WR 6026 (free base) and 1.21 ng/ml for WR 211,789 (free base) for blood and plasma. The coefficients of variation of the inter- and intraday assay precision analyses were less than 10% at all concentrations. The method is simple, precise, more sensitive, and includes the capability of quantitating WR 211,789 (free base) as well as the parent drug compared to earlier methods.

Study Report 19: Mefloquine in Human Blood

Study Characteristics: Study Report 19

Test Article:	Mefloquine
Test System:	human blood
Internal Standard:	chlorpheniramine
Sample Assay Volume:	0.5 ml
Sample Cleanup:	pentane/methylene chloride (7:3, v/v) extraction

Analytical System

Detector:	UV at 280 nm
Column Type:	silica
Column Size:	4.6x250 mm, 5µ particle size
Mobile Phase:	methanol/water (4:1, v/v) final concentration of 5 mM $(NH_4)_2HPO_4$ at pH 7.5

Validation Results: Mefloquine in blood

Quantitation Limit:	7.36 ng/ml
Standard curve range:	7.36-2210 ng/ml
Interday Precision	
Concentration Range:	14.7-1472 ng/ml
CV Range:	3.94-8.41%
Intraday Precision	
Concentration Range:	14.7-1472 ng/ml
CV Range:	2.74-10.9%
Blind Sample Assay	
Concentration Range:	11.52-1536 ng/ml
Bias Range:	-12.6 to +7.20%
Mean Recovery:	91.5%
Stable Blood Storage:	-20°C for 4 months

Study Description: Mefloquine in Human Blood (the methodology was presented in DAMD17-92-C-2028 mid-term report)

Mefloquine (hydrochloride), (WR 142,490: erythro-a-(2-piperidyl)-2,8-bis(trifluoromethyl)-4-quinolinemethanol hydrochloride), is an alternative curative agent for the treatment of falciparum malaria.[18,19] Mefloquine (hydrochloride) has also been shown to prophylactically suppress mosquito induced infections by *Plasmodium vivax* and *P. falciparum* in human volunteers.[20,21] Published plasma and/or blood analytical methods employ gas-liquid chromatography (GLC)[22,23,24] thin layer chromatography (TLC),[25] gas chromatography-mass spectrometry (GC-MS)[26] or high performance liquid chromatography (HPLC) (Study Reports 6 and 14).[27,28,29,30,31,32,33] The GLC

methods require derivitization, and sample volume in the method described by Nakagawa, *et al.* uses 5 ml samples. The TLC method has no internal standard and is insufficiently sensitive. The GC-MS method requires derivitization and the increased expense of mass spectrometry.

Many HPLC have been reported. The method reported by Grindel, *et al.*, required three times extraction from 5 ml plasma samples and, upon solvent evaporation, the residues required overnight storage in a vacuum desiccator. Kapetanovic, *et al.* used a 3 step extraction of 1 ml samples. Our earlier study (Study Report 6) described a protein precipitation method for 0.2 ml plasma samples. Franssen, *et al.*, described a method for plasma and blood analysis for mefloquine and its carboxylic acid metabolite with 50 ng/ml mefloquine and 100 ng/ml metabolite detection limits. Karbwang, *et al.*, described a 50 ng/ml detection limit, 100 ng/ml quantitation limit method for mefloquine in blood and plasma. Coleman, *et al.*, measured mefloquine at 10 ng/ml in liver perfusate. Riviere *et al.*, presented a method with a 20 ng/ml detection limit and 100 ng/ml quantitation limit in plasma. Bergqvist, *et al.*, describe two HPLC methods for determination of mefloquine and its principal metabolite in plasma and blood, the first with 30 ng/ml plasma and 150 ng/ml blood quantitation limits and the second with 75 ng/ml quantitation limits for both compounds.

We reported (Study Report 14) the development of a simple and rapid HPLC assay for mefloquine (free base) that requires 0.5 ml plasma samples and a one step extraction, has an 7.36 ng/ml quantitation limit and produces chromatograms with a cleaner baseline than our previous method. Study Report 19 describes the extension of our plasma method to include analysis of blood samples. Study Report 19 also describes status of steps taken toward extension of the method for determination of the main mefloquine metabolite, WR 160,972 (2,8-bis-(trifluoromethyl)-4-quinoline carboxylic acid).[34]

The blood method was modified from the plasma method described in Study Report 14, "Quantitation of Mefloquine (Free Base) in Plasma by High-Performance Liquid Chromatography, Extraction Method." The blood method primarily differs from the plasma method in sample preparation by:

1. Allowing blood standard curve calibrator samples to equilibrate for 1 hour following spiking with mefloquine working solutions;

2. Addition of 0.5 ml water; and

3. Sonication for 10 min prior to addition of internal standard.

Blood samples for analysis are pipetted (0.5 ml) into screw top tubes. Add 100 µl of a saturated solution of sodium carbonate and vortex the mixture for 1 min. Then, add 100 µl of the internal standard working solution (CPA, 12 µg/ml) and vortex the mixture for 1 min. The sample is extracted with 5 ml of pentane/methylene chloride (7:3, v/v), evaporated to dryness under nitrogen, resuspended in 200 µl of mobile phase and injected (40-80 µl) onto the HPLC column.

An addendum with blind sample results enclosed with Quarterly Report 6 completed Study Report 19 (Status Report, dated January 14, 1992 and titled "Quantitation of Mefloquine (Free Base) in Blood by High-Performance Liquid Chromatography, Extraction Method." Further work on this assay is scheduled to include WR 160,972 method development, but work on this aspect of the project has been assigned a low priority by the COR.

Study Report 21: *p*-Aminoheptanophenone and Metabolites in Dog Plasma and Rat Plasma

Study Characteristics: Study Report 21	
Test Article:	WR 269,410 (*p*-aminoheptanophenone)
Test System:	dog plasma
Internal Standard:	WR 258,948 (*p*-aminooctanophenone)
Sample Assay Volume:	0.5 ml
Sample Cleanup:	methyl *t*-butyl ether extraction
Analytical System	
Detector:	UV at 316 nm
Column Type:	C18 bonded silica
Column Size:	4.6x250 mm, 5µ particle size
Mobile Phase:	acetonitrile/water (1:1, v/v) and 0.15% H_3PO_4
Validation Results:	
Quantitation Limit:	4.08 ng/ml
Standard curve range:	4.08-816 ng/ml
Blind Sample Assay	see Appendix A, DAMD17-92-C-2028 Midterm Report

Study Description: *p*-Aminoheptanophenone and Metabolites in Dog Plasma and Rat Plasma (the analytical procedure was presented in DAMD17-92-C-2028 final report)

Method validation will be reported in Study Report 21 (preparation in progress covering *p*-aminoheptanophenone (PAHP, WR 269,410) *p*-aminooctanophenone (PAOP, WR 258,948) and *p*-aminopropiophenone (PAPP, WR 302)). Results from the analysis of blind spiked (by WRAIR) dog plasma samples were presented in Appendix A of the mid-term report.

Study Report 22: WR 6026 and Metabolites in Human Urine

Study Characteristics: Study Report 22

Test Article:	WR 6026 WR 211,789 WR 254,421
Test System:	human urine
Internal Standard:	verapamil
Sample Assay Volume:	0.5 ml
Sample Cleanup:	methyl *t*-butyl ether extraction

Analytical System

Detector:	UV at 350 nm
Column Type:	silica
Column Size:	4.6x250 mm, 5µ particle size
Mobile Phase:	acetonitrile/0.0075% phosphoric acid (80:20, v/v) at pH 6.9.

Validation Results WR 6026 free base

Quantitation Limit:	5.17 ng/ml
Interday CV:	14.8%
Interday Error:	2.44%
Standard curve range:	2.17-414 ng/ml
Interday Precision	
Concentration Range:	10.4-259 ng/ml
CV Range:	3.90-7.42%
Intraday Precision	
Concentration Range:	10.4-259 ng/ml
CV Range:	3.83-28.4%
Blind Sample Assay	
Concentration Range:	5.20-101.2 ng/ml
Bias Range:	-10.6 to +33.9%
Mean Recovery:	97.2%
Stable Plasma Storage:	-70°C for 4 months
Stable Prepared Sample:	Room temp. for 48 hours

Validation Results WR 211,789 free base

Quantitation Limit:	509 ng/ml
Interday CV:	14.7%
Interday Error:	4.55%
Standard curve range:	5.09-407 ng/ml
Interday Precision	
Concentration Range:	10.2-255 ng/ml
CV Range:	4.07-10.0%
Intraday Precision	
Concentration Range:	10.2-255 ng/ml
CV Range:	5.12-23.3%
Blind Sample Assay	
Concentration Range:	5.20-102.6 ng/ml
Bias Range:	-11.8 to +33.9%
Mean Recovery:	92.8%
Stable Plasma Storage:	-70°C for 4 months
Stable Prepared Sample:	Room temp. for 48 hours

Validation Results WR 254,421 free base

Quantitation Limit:	45.4 ng/ml
Interday CV:	7.51%
Interday Error:	1.60%
Standard curve range:	45.4-3630 ng/ml
Interday Precision	
Concentration Range:	90.8-2270 ng/ml
CV Range:	3.09-5.86%
Intraday Precision	
Concentration Range:	90.8-2270 ng/ml
CV Range:	3.55-10.0%
Blind Sample Assay	
Concentration Range:	50.1-979.4 ng/ml
Bias Range:	-10.7 to +9.63%
Mean Recovery:	94.2%
Stable Plasma Storage:	-70°C for 4 months
Stable Prepared Sample:	Room temp. for 48 hours

Study Description: WR 6026 and Metabolites in Human Urine (the methodology was presented in DAMD17-92-C-2028 final report)

Study Report 22 "Quantitation of WR 6026, WR 211,789 and WR 254,421 (as Free Bases) in Human Urine by High Performance Liquid Chromatography,"

was submitted for review July 18, 1994. This method is a modified version of the plasma method.

WR 6026 (dihydrochloride) (6-methoxy-8-(6-diethyl amino hexyl amino) lepidine dihydrochloride) (Figure 1), is a very effective antileishmanial drug in hamsters infected with *Leishmania donovani* .[16] Because antimony compounds are not always effective and the other drugs in use have toxic effects,[17,18] alternative therapies are needed. Since WR 6026 (dihydrochloride) is a likely candidate and since WR 6026 (dihydro-chloride) is scheduled for clinical testing, it is extremely important to develop an analytical method capable of measuring concentrations of WR 6026 (free base) at nanogram per milliliter concentrations in biological samples. This report describes an assay developed to determine the concentrations (as free bases) of WR 6026 and of its metabolites, WR 211,789 (6-methoxy-8-(6-ethyl-aminohexylamino) lepidine dihydrochloride, hemihydrate) and WR 254,421 (8-(6'-N,N-diethylaminohexyl)amino-4-hydroxymethyl-6-methoxyquinoline, dihydrochloride) in urine. WR 211,789 has been detected in a rat microsomal preparation.[21] This assay adds the capability of detection of WR 6026, WR 211,789 and WR 254,421 (as free bases) in urine to earlier assays for WR 6026 and WR 211,789 in plasma and blood.

Assay samples were prepared by spiking known volumes of human urine with a known amount (constant over all samples) of the verapamil internal standard (IS). Standard curve samples were generated by spiking interference free human urine samples with known amounts of WR 6026, WR 211,789 and WR 254,421 (as free bases) and IS. These standard curve and assay samples were extracted, then injected onto an HPLC column for separation and subsequent ultraviolet detection. The peak height ratios of WR 6026, WR 211,789 and WR 254,421 (as free bases) to IS were calculated for each sample from the measured peak heights obtained by HPLC. Finally, standard curve concentrations and WR 6026, WR 211,789 and WR 254,421 (as free bases) to IS peak height ratios of the standard curve samples were fit by least squares linear regression to the equation for the best straight line ($y = mx + b$, where y = peak height ratio and x = WR 6026, WR 211,789 or WR 254,421 (as free bases) concentrations), and drug concentrations in assay samples were calculated by this equation from the WR 6026, WR 211,789 and WR 254,421 (as free bases) to IS peak height ratios obtained by HPLC.

Sample volume taken for analysis was 0.5 ml of urine. A constant amount, approximately 5 µg, of the internal standard, verapamil, was added to and mixed with each sample. Next, 100 µl of 1 N NaOH was added to and mixed with each sample. Then, samples were extracted with 5 ml of methyl *t*-butyl ether. The extraction solution was transferred to a second culture tube, evaporated to dryness under nitrogen, and reconstituted in 200 µl of mobile phase. Finally 20-160 µl of the sample was injected onto the HPLC column.

In typical chromatograms for blank urine and urine samples spiked with WR 6026, WR 211,789 or WR 254,421, WR 6026, WR 211,789 or WR 254,421 eluted at 15.3, 14.3, and 18.2 minutes, respectively, and the internal standard eluted at 12.4

minutes. The coefficients of determination for WR 6026, WR 211,789 or WR 254,421 interday and intraday precision standard curves were 0.9825 or higher.

The reversed-phase system (alkyl bonded silica gel with an aqueous mobile phase) is the most widely used HPLC technique in assays for drugs in biological fluids. In this kind of a system, the retention mechanism depends mainly on the lipophilic character of substances to be analyzed. Such a mechanism also retains considerable amounts of other lipophilic substances, thereby interfering with the drug peak. On the other hand, in a system consisting of a bare silica gel and an aqueous mobile phase, the retention mechanism results mainly from ion exchange and only partially from lipophilic interactions. Thus, endogenous non-ionic neutral lipid compounds and anionic compounds will not be retained on the silica gel column; only the cationic (e.g. ammonium) ions will be retained. The interfering substances in biological fluids elute at the solvent front, leaving a very clean baseline around the retention time of the drug.

HPLC assays for basic amine drugs in biological samples that make use of a silica gel column and an aqueous mobile phase have been operated in this laboratory for over 5 years. By use of an organic solvent extraction step for sample clean-up, an unbonded silica gel column combined with an aqueous mobile phase for separation, and the superior capability of ultraviolet detection, the free base concentrations of WR 6026, WR 211,789 and WR 254,421 can be quantitatively and reliably measured in human urine samples. The assay described in the report dated July, 18, 1994, requires 0.5 ml urine samples to determine the free base concentrations of WR 6026, WR 211,789 or WR 254,421. The method involves sample cleanup with a methyl *t*-butyl ether extraction, separation on an unbonded silica gel column (5 μm particle size) run with an aqueous mobile phase, and ultraviolet detection. The minimum quantitation limits of the assay are 5.17, 5.09, and 45.4 ng/ml for WR 6026, WR 211,789 and WR 254,421 free base, respectively, with a signal to noise ratio of 3 to 1. Average mean recoveries over the working range of the standard curve were 97.2, 92.8, and 94.2 percent for WR 6026, WR 211,789 and WR 254,421 free base, respectively. The respective percent coefficients of variation (CVs) of the inter- and intraday assay precision analysis for the free base concentrations of WR 6026 ranged from 3.90% to 7.42% and 3.83% to 28.4%; of WR 211,789 ranged from 4.07% to 10.0% and 5.12% to 23.3%; and of WR 254,421 ranged from 3.09% to 5.86% and 3.55% to 10.0%. No discernible pattern of degradation was observed in long term or autosampler stability tests.

Study Report 26: WR 242511 in Human and Dog Plasma

Study Characteristics: Study Report 26

Test Article:	WR 242511
Test System:	human plasma dog plasma
Internal Standard:	Chlorpheniramine maleate
Sample Assay Volume:	0.5 ml
Sample Cleanup:	methyl *t*-butyl ether extraction

Analytical System

Detector:	UV at 350 nm
Column Type:	silica
Column Size:	4.6x250 mm, 5µ particle size
Mobile Phase:	CH_3CN/H_2O (7:3, v/v) with 0.008% TEA and 0.005% H_3PO_4 (final concentrations)

Validation Results: WR 242511 free base in human plasma

Lower Limit of Quantitation:	4.00 ng/ml
Interday Mean, CV and RE:	4.57 ng/ml, 5.84% and 14.2%
Intraday Mean, CV and RE:	3.69 ng/ml, 7.87% and -15.6%
Standard curve range:	4.00 to 1024 ng/ml
Interday Precision Concentrations:	8.00, 32.0, 128, and 256 ng/ml
CV Range:	8.74 to 11.9%
RE Range:	-3.32 to +5.40%
Intraday Precision Concentrations:	8.00, 32.0, 128, and 256 ng/ml
CV Range:	2.99 to 5.90%
RE Range:	+5.21 to +12.3%
Blind Sample Assay	
Concentration Range:	4.70 to 822 ng/ml
RE Range:	-6.27 to +21.9%
Overall Mean Recovery:	77.1%
Stability	
Plasma Freezer Storage:	-70°C for 6 months -20°C for 6 months
Processed Sample:	Room temp. for 4 days
Plasma Storage:	Room temp. for 6 hours
5 Cycle Freeze/Thaw:	5 cycles to -70°C
Standard Solution:	6 months

Short Validation Results: WR 242511 free base in dog plasma

Lower Limit of Quantitation:	4.00 ng/ml
Precision Mean, CV and RE:	4.42 ng/ml, 6.52% and +10.4%
Standard curve range:	4.00 to 1024 ng/ml
Interday Precision Concentrations:	8.00, 32.0, 128, and 256 ng/ml
CV Range:	4.14 to 13.3%
RE Range:	-5.78 to -0.104%
Intraday Precision Concentrations:	8.00, 32.0, 128, and 256 ng/ml
CV Range:	0.764 to 5.12%
RE Range:	-10.8 to -3.35%
Overall Mean Recovery:	79.3%

Study Description: WR 242511 in Human and Dog Plasma (the methodology was presented in DAMD17-92-C-2028 final report)

This report describes a high performance liquid chromatographic (HPLC) assay and provides data validating the assay for a compound of the 8-aminoquinoline class. The compound, 8-[(4-amino-1-methylbutyl)amino]-5-(1-hexyloxy)-6-methoxy-4-methylquinoline (DL) tartrate (WR 242511, Figure 1), holds promise[35] in an effort to replace primaquine, the radical cure and prophylaxis for vivax and ovale malaria and is being developed by WRAIR as an anti-cyanide drug.

Assays for other 8-aminoquinolines include high performance liquid chromatography (HPLC) methods with electrochemical,[36] ultraviolet,[37] and fluorescence[38] detection. An HPLC method with oxidative electrochemical detection has been described[39] for WR 242511 in 0.25 ml plasma samples with a detection limit of 10 ng/ml. This report presents validation data for a superior method that employs an aqueous mobile phase, an unbonded silica gel column[40] and ultraviolet detection for WR 242511 free base concentration determinations in 0.5 ml human and dog plasma samples with a lower limit of quantitation of 4 ng/ml.

Plasma samples were analyzed for WR 242511 free base with an HPLC procedure that uses a silica gel column, an (acetonitrile/water) aqueous mobile phase, UV absorbance detection, and a 0.5 ml method sample size. Sample cleanup consisted of extraction into methyl *t*-butyl ether. The methodology contains detailed procedures, which are summarized below.

Assay samples were prepared by spiking known volumes of human plasma with a known amount (constant over all samples in a run) of CPA internal standard (IS). Standard curve samples were generated by spiking a known amount of WR 242511 tartrate into interference free human plasma which is then brought to a known volume, divided by serial dilution and spiked with a known amount of IS. These standard curve and assay samples were prepared for analysis, then 40 µl aliquots were injected onto the HPLC column for

chromatographic separation and subsequent UV absorbance detection of drug and IS peaks. The peak height ratios of WR 242511 to IS were calculated for each sample from the measured peak heights obtained by HPLC. Next, standard curve concentrations and WR 242511 to IS peak height ratios of the standard curve samples were fit by 1/y weighted least squares linear regression to the equation for the best straight line, y = mx + b, where y = peak height ratio and x = WR 242511 free base concentrations. Finally, drug concentrations in assay samples were calculated for each run by this equation from the WR 242511 to IS peak height ratios obtained by HPLC.

Stock solutions of WR 242511 tartrate and chlorpheniramine maleate internal standard (IS) were stored in a 4°C refrigerator and protected against exposure to light as necessary, and checked for deterioration by following the ratio of drug to internal standard peak heights in a diluted solution (solutions are discarded when a more than 10% change in the ratio is observed or by 2 months after the preparation date).

Plasma samples for analysis were thawed and mixed by vortexing (if appropriate), then pipetted (0.5 ml) into glass culture tubes. A constant amount (1.0 µg chlorpheniramine maleate) of IS, 100 µl of 0.1N NaOH, and 3 ml of methyl *t*-butyl ether are added. Upon centrifugation and freezing of the aqueous layer, the resulting supernatant was transferred to a clean tube, evaporated to dryness, reconstituted in 70% acetonitrile, transferred to WISP vials, and injected onto the column.

The assay described in the report dated December, 12, 1996, requires 0.5 ml plasma samples to determine the concentrations of WR 242511 free base. The method involves extraction from plasma with methyl *t*-butyl ether, separation on a silica gel column with an aqueous mobile phase in an isocratic elution, and ultraviolet absorbance detection. The advantages of this method include a clean baseline and a short run time.

The reversed-phase system (alkyl bonded silica gel with an aqueous mobile phase) is the most widely used HPLC technique in assays for drugs in biological fluids. In this kind of a system, the retention mechanism depends mainly on the lipophilic character of substances to be analyzed. Such a mechanism also retains considerable amounts of other lipophilic substances, thereby interfering with the drug peak. On the other hand, in a system consisting of a bare silica gel and an aqueous mobile phase, the retention mechanism results mainly from ion exchange[6] and only partially from lipophilic interactions. Thus, endogenous non-ionic neutral lipid compounds and anionic compounds will not be retained on the silica gel column; only the cationic (e.g. ammonium) ions will be retained. The interfering substances in biological fluids elute at the solvent front, leaving a very clean baseline around the retention time of the drug. In this method, the mobile phase is recycled through a non analytical silica gel column overnight to saturate it with silica. Overnight saturation of mobile phase prior to use is beneficial to the whole system, since silica gel slowly dissolves in neutral aqueous solution and the water flowing through the silica gel approaches the equilibrium concentration of silica. The saturated mobile phase does not

dissolve silica from the analytical columns and degradation of the analytical column is decreased relative to the single pass system.

By use of a solvent extraction step for sample clean-up, an unbonded silica gel column combined with an aqueous mobile phase for separation, and the superior capability of ultraviolet detection, the concentration of WR 242511 free base can be quantitatively and reliably measured in human and dog plasma samples. The drug and IS are baseline separated, and no interfering peaks were observed. The assay was demonstrated to be linear within the range of the standard curve, 4.00 to 1024 ng/ml WR 242511 free base. The CVs of results for human plasma precision validation ranged from 8.74 to 11.9% interday and 2.99 to 5.90% intraday, while percent RE of measured results compared to serially diluted concentrations ranged -3.32 to +5.40% interday and +5.21 to +12.3% intraday for WR 242511 free base. The mean concentrations (n = 6) obtained for human plasma samples serially diluted to the LLOQ (4.00 ng/ml) were 4.57 ng/ml interday (5.84% CV and +14.2% RE) and 3.38 ng/ml intraday (7.87% CV and -15.6% RE) where the signal to noise ratio was better than 3 to 1. WR 242511 average recovery from human plasma extraction for the four concentrations within the standard curve quantitation limits was 77.1%. Stability test results indicate WR 242511 is sufficiently stable in 1) human plasma samples prepared for assay (includes extraction, evaporation, and reconstitution in 70% acetonitrile) to withstand room temperature (RT) storage for at least 4 days, 2) human plasma at -70°C and at -20°C to permit storage without significant degradation for up to 6 months, 3) human plasma to withstand RT storage for at least 6 hours without significant degradation, and 4) human plasma to withstand 5 cycles of repeated freezing in a -70°C freezer and thawing at room temperature without significant degradation. The CVs of the results for the analyses of blind WR 242511 human plasma samples (n = 5) at five concentrations within the standard curve limits ranged 0.958-9.61% while R.E.s ranged -8.39 to +21.9%.

The CV (and corresponding RE, for n = 6) results of dog plasma precision validation for WR 242511 ranged from 4.14 to 13.3% (-5.78 to -0.104%) interday and 0.764 to 5.12% (-10.8 to -3.35%) intraday. Mean back calculated dog plasma concentration results of replicate analyses of precision standard curve samples serially diluted to the LLOQ (4.00 ng/ml for WR 242511 free base concentration) was 4.42 ng/ml (6.52% CV and +10.4% RE, n = 4). The signal to noise ratio was better than 3 to 1 for these LLOQ samples. WR 242511 average recovery from dog plasma extraction for the four concentrations within the standard curve quantitation limits was 79.3%.

Study Report 28: Halofantrine and WR 178460 R&S Isomers in Human Plasma

Study Characteristics: Study Report 28

Test Article:	Halofantrine (+ isomer) Halofantrine (- isomer) WR 178460 (+ isomer) WR 178460(- isomer)
Test System:	human plasma
Internal Standard:	Desmethylimipramine
Sample Assay Volume:	0.5 ml
Sample Cleanup:	protein precipitation by CH_3CN, then basic methyl *t*-butyl ether extraction

Analytical System

Detector:	fluorescence: Ex-300 nm; Em-375 nm
Column Type:	chiral AD
Column Size:	4.6x250 mm, 5µ particle size
Mobile Phase:	Hexane, Ethanol, 2-butanol, diethylamine (100:1.5:1.3:0.1, v/v/v/v) (final concentrations)

Validation Results: +Halofantrine isomer in human plasma

Lower Limit of Quantitation:	10.0 ng/ml
Interday Mean, CV and RE:	10.1 ng/ml, 6.08%, and +0.617%
Intraday Mean, CV and RE:	11.6 ng/ml, 8.59%, and +15.8%
Standard curve range:	10.0 to 400 ng/ml
Interday Precision Concentrations:	20.0, 50.0, 100, and 300 ng/ml
CV Range:	1.92% to 6.41%
RE Range:	-4.04% to -0.222%
Intraday Precision Concentrations:	20.0, 50.0, 100, and 300 ng/ml
CV Range:	0.799% to 3.45%
RE Range:	-4.22% to +0.556%
Overall Mean Recovery:	73.6%
Stability	
Processed Sample:	Room temp. for 48 hours
Processed Sample:	4°C for 48 hours -20°C for 72 hours

Validation Results: -Halofantrine isomer in human plasma

Lower Limit of Quantitation:	10.0 ng/ml
Interday Mean, CV and RE:	10.1 ng/ml, 10.5%, and +1.00%
Intraday Mean, CV and RE:	11.1 ng/ml, 7.03%, and +10.5%
Standard curve range:	10.0 to 400 ng/ml
Interday Precision Concentrations:	20.0, 50.0, 100, and 300 ng/ml
CV Range:	2.10% to 4.27%
RE Range:	-3.13% to +1.75%
Intraday Precision Concentrations:	20.0, 50.0, 100, and 300 ng/ml
CV Range:	0.854% to 2.01%
RE Range:	-3.65% to +1.90%
Overall Mean Recovery:	82.6%
Stability	
Processed Sample:	Room temp. for 48 hours
Processed Sample:	4°C for 48 hours
	-20°C for 72 hours

Validation Results: +WR178460 isomer in human plasma

Lower Limit of Quantitation:	15.0 ng/ml
Interday Mean, CV and RE:	15.5 ng/ml, 3.68%, and +3.44%
Intraday Mean, CV and RE:	14.8 ng/ml, 13.2%, and -1.56%
Standard curve range:	15.0 to 600 ng/ml
Interday Precision Concentrations:	30.0, 75.0, 150, and 450 ng/ml
CV Range:	4.89% to 6.79%
RE Range:	+1.58% to +3.78%
Intraday Precision Concentrations:	30.0, 75.0, 150, and 450 ng/ml
CV Range:	2.24% to 4.69%
RE Range:	-0.778% to +2.11%
Overall Mean Recovery:	95.5%
Stability	
Processed Sample:	Room temp.: not stable at 18 hours
Processed Sample:	4°C for 48 hours
	-20°C for 72 hours

Validation Results: -WR178460 isomer in human plasma

Lower Limit of Quantitation:	15.0 ng/ml
Interday Mean, CV and RE:	14.9 ng/ml, 7.64%, and -0.444%
Intraday Mean, CV and RE:	14.4 ng/ml, 11.5%, and -4.33%
Standard curve range:	15.0 to 600 ng/ml
Interday Precision Concentrations:	30.0, 75.0, 150, and 450 ng/ml
CV Range:	5.53% to 7.52%
RE Range:	+1.50% to +3.69%
Intraday Precision Concentrations:	30.0, 75.0, 150, and 450 ng/ml
CV Range:	1.79% to 4.16%
RE Range:	-8.44% to 2.22%
Overall Mean Recovery:	98.8%
Stability	
Processed Sample:	Room temp.: not stable at 18 hours
Processed Sample:	4°C for 48 hours
	-20°C for 72 hours

Study Description: Short Validation of a HPLC Method for the Determination of R & S Isomers of Halofantrine and WR 178460 in Human Plasma Samples

This project was requested as described in a COR letter dated Oct. 20, 1995. WR 216062 and WR 216063 standard samples were received October 20, 1995 for use in development and validation of an assay for halofantrine and desbutylhalofantrine enantiomers in human plasma. A draft report was submitted February 9, 2001.

This report describes the analytical method and validation of the analytical method used to measure concentrations of R & S isomers of halofantrine and WR 178460 in human plasma samples. The method was developed and validated at the Analytical Division, Drug Studies Unit (DSU), UCSF, San Francisco, CA. The method was requested by the Contracting Officer's Representative at the Walter Reed Army Institute of Research (WRAIR) for contract DAMD17-97-C-7058.

Method Summary: Human plasma samples (0.5 ml) were analyzed for R & S isomers of halofantrine and WR 178460 with an HPLC procedure that uses a fluorescence detector, a chiral column, a hexane/ethanol/2-butanol/diethylamine (100:1.5:1.3:0.1, v/v/v/v) mobile phase, and an HP integrator. Sample cleanup consisted of precipitation with acetonitrile and extraction into methyl *t*-butyl ether prior to separation by HPLC.

Validation Results Summary: The following validation parameters were evaluated for R & S isomers of halofantrine and WR 178460.

1. Specificity: No significant (i.e., above the normal noise level) endogenous interfering peaks for R & S isomers of halofantrine and WR 178460 or for the internal standard were observed in blank human plasma.

2. Inter-Day Precision and Accuracy: Interday precision and accuracy measurements were determined by analyzing quality control (QC) samples made of human plasma spiked with known amounts of drug and metabolite. Each of 6 sets (n=2) of control samples at 4 different drug concentrations was evaluated (6 standard curves for the each isomer were run). Precision coefficients of variation (CV), ranged from 2.10% to 4.27% for the (-) isomer of halofantrine, 1.92% to 6.41% for the (+) isomer of halofantrine, 5.53% to 7.52% for the (-) isomer of WR 178460, and 4.89% to 6.79% for the (+) isomer of WR 178460. The accuracy, defined by the relative error (RE) ranged from -3.13% to +1.75% for the (-) isomer of halofantrine, -4.04% to -0.222% for the (+) isomer of halofantrine, +1.50% to +3.69% for the (-) isomer of WR 178460, and +1.58% to +3.78% for the (+) isomer of WR 178460.

3. Intra-Day Precision and Accuracy: Intraday precision and accuracy measurements were determined by analyzing quality control (QC) samples made of human plasma spiked with known amounts of drug and metabolite. For intraday precision, 6 sets (n=1) of control samples for each of 4 different drug concentrations were evaluated with 1 standard curve for each isomer on the same run. CVs ranged from 0.854% to 2.01% for the (-) isomer of halofantrine, 0.799% to 3.45% for the (+) isomer of halofantrine, 1.79% to 4.16% for the (-) isomer of WR 178460, and 2.24% to 4.69% for the (+) isomer of WR 178460. REs ranged from -3.65% to +1.90% for the (-) isomer of halofantrine, -4.22% to +0.556% for the (+) isomer of halofantrine, -8.44% to 2.22% for the (-) isomer of WR 178460, and -0.778% to +2.11% for the (+) isomer of WR 178460.

4. Lower Limit of Quantitation (LLOQ): The LLOQs for this assay are equivalent to the low points of the standard curves, or 10.0 ng/ml for the R & S isomers of halofantrine and 15.0 ng/ml for the R & S isomers of WR 178460. Interday mean, CV, and RE results were: 10.1 ng/ml, 10.5%, and +1.00% for the (-) isomer of halofantrine; 10.1 ng/ml, 6.08%, and +0.617% for the (+) isomer of halofantrine; 14.9 ng/ml, 7.64%, and -0.444% for the (-) isomer of WR 178460; and 15.5 ng/ml, 3.68%, and +3.44% for the (+) isomer of WR 178460. Intraday mean, CV, and RE results were: 11.1 ng/ml, 7.03%, and +10.5% for the (-) isomer of halofantrine; 11.6 ng/ml, 8.59%, and +15.8% for the (+) isomer of halofantrine; 14.4 ng/ml, 11.5%, and -4.33% for the (-) isomer of WR 178460; and 14.8 ng/ml, 13.2%, and -1.56% for the (+) isomer of WR 178460.

5. Linear Range: The validated linear concentration ranges for this assay were 10.0 to 400 ng/ml for the R & S isomers of halofantrine and 15.0 to 600 ng/ml for the R & S isomers of WR 178460. Precision standard curve CVs, ranged from 1.00% to 10.5% for the (-) isomer of halofantrine, 1.38% to 7.09% for the (+) isomer of halofantrine, 1.15% to 8.68% for the (-) isomer of WR 178460, and 1.15% to 5.71% for the (+) isomer of WR 178460. REs ranged from -1.42% to +4.86% for the (-) isomer of halofantrine, -2.17% to +3.90% for the (+) isomer of halofantrine, -4.11% to 3.60% for the

(-) isomer of WR 178460, and -5.89% to +3.44% for the (+) isomer of WR 178460.

6. Recovery: Peak area ratios of processed samples to unprocessed samples provided an overall recoveries of 82.6% for the (-) isomer of halofantrine, 73.6% for the (+) isomer of halofantrine, 98.8% for the (-) isomer of WR 178460, and 95.5% for the (+) isomer of WR 178460.

7. Stability:

   a. Processed Sample – Ambient temperature: R & S isomers of halofantrine were shown to be stable up to 48 hours at ambient temperature and R & S isomers of WR 178460 were shown to be not stable by 18 hours at ambient temperature.

      Processed Sample - Refrigerated: R & S isomers of halofantrine and WR 178460 were shown to be stable up to 48 hours at -4°C and up to 72 hours at -20°C.

Analytical Method

Human plasma samples (0.5 ml) were analyzed for R & S isomers of halofantrine and WR 178460 with an HPLC procedure that uses a fluorescence detector, a chiral column (4.6 x 250 mm, 5 µm particle size), a hexane/ethanol/2-butanol/diethyl-amine (100:1.5:1.3:0.1, v/v/v/v) mobile phase, and an HP integrator. Sample preparation consisted of addition of 150 µl of desmethylimipramine internal standard (IS) methanol solution, precipitation with 1 ml of acetonitrile (followed by centrifugation for 1 minute and transfer and evaporation to 0.2 ml of the supernatant), followed by extraction with 5 ml methyl *t*-butyl ether, evaporation of the organic layer and reconstitution in mobile phase prior to injection onto the HPLC column. Standard curve and quality control (QC) samples were generated by spiking interference free human plasma samples with known amounts of R & S isomers of halofantrine and WR 178460 and IS. Standard curve, QC and assay samples were prepared as described, then 10-35 µl aliquots were injected into the HPLC system for chromatographic separation and subsequent fluorometric detection. The peak height ratios of R & S isomers of halofantrine and WR 178460 to IS were calculated for each sample from the measured peak heights obtained by HPLC. Finally, spiked concentrations and R or S isomer of halofantrine or WR 178460 to IS peak height ratios of the standard curve samples were fit by 1/y weighted least squares linear regression to the equation for the best straight line ($y = mx + b$, where y = peak height ratio and x = R or S isomers of halofantrine or WR 178460 concentrations), and drug concentrations in assay samples were calculated by these equation from the R or S isomer of halofantrine or WR 178460 to IS peak height ratios obtained by HPLC.

Calibration standards and validation samples with drug concentrations within the calibration range of the assay were analyzed to assess the performance of the

assay. Calibration standards and validation samples were generated by spiking blank human plasma with racemic halofantrine and WR 178460.

Retention times (approximate, in decimal minutes) were 30.0: internal standard, 17.2: (+)halofantrine, 18.5: (-)halofantrine, 34.3: (-)WR 178460, and 38.8: (+)WR 178460.

Conclusion

The HPLC method for analysis of human plasma to determine concentrations of R & S isomers of halofantrine and WR 178460 was validated for the concentration ranges of 10.0 ng/ml to 400 ng/ml for R & S isomers of halofantrine and 15.0 ng/ml to 600 ng/ml for R & S isomers of WR 178460. Preparation time for a run of 40 sponsor samples plus standard curve and control samples is about 1&1/2 hours. Run time is about 48 hours. The method was demonstrated to be precise, accurate, and sufficiently reproducible for analysis of study samples.

Study Report 29A: Chloroquine and Monodesethylchloroquine in Human Blood

Study Characteristics: Study Report 29A

Test Article:	Chloroquine Monodesethylchloroquine
Test System:	human blood
Internal Standard:	Neostigmine bromide
Sample Assay Volume:	100 µl
Sample Cleanup:	Lyse cells with water, precipitate proteins with acetonitrile

Analytical System

Detector:	MS/MS
Column Type:	Hypersil silica
Column Size:	4.6x50 mm, 3 µ particle size
Mobile Phase:	CH_3CN/H_2O (9:1, v/v) with 0.1% TFA and 5mM ammonium acetate

Validation Results: Chloroquine free base in human blood

Lower Limit of Quantitation:	20 ng/ml
Interday Mean, CV and RE:	16.9 ng/ml, 5.93% and –15.4%
Intraday Mean, CV and RE:	22.6 ng/ml, 9.81% and +12.9%
Standard curve range:	20 to 2000 ng/ml
Interday Precision Concentrations:	40, 100, 250, and 1500 ng/ml
CV Range:	7.57% to 11.4%
RE Range:	-7.49 to –1.99 %
Intraday Precision Concentrations:	40, 100, 250, and 1500 ng/ml
CV Range:	2.04 to 8.22 %
RE Range:	-8.08 to +0.210%
Blind Sample Assay	
Concentration Range:	Not determined
RE Range:	Not determined
Overall Mean Recovery:	70.4%
Stability	
Plasma Freezer Storage:	Not determined
Processed Sample:	4°C for 27 days
Plasma Storage:	Room temp. for 6 hours
5 Cycle Freeze/Thaw:	5 cycles to -20°C
Standard Solution:	Not determined

Validation Results: Monodesethylchloroquine free base in human blood

Lower Limit of Quantitation:	20 ng/ml
Interday Mean, CV and RE:	17.8 ng/ml, 11.0%, and -10.8
Intraday Mean, CV and RE:	22.2 ng/ml, 8.34%, and +11.2%
Standard curve range:	20 to 2000 ng/ml
Interday Precision Concentrations:	40, 100, 250, and 1500 ng/ml
CV Range:	8.51% to 13.0%
RE Range:	-8.28% to -4.87%
Intraday Precision Concentrations:	40, 100, 250, and 1500 ng/ml
CV Range:	2.62% to 9.84%
RE Range:	-11.4% to +0.103%
Blind Sample Assay	
Concentration Range:	Not determined
RE Range:	Not determined
Overall Mean Recovery:	61.3%
Stability	
Plasma Freezer Storage:	Not determined
Processed Sample:	4°C for 27 days
Plasma Storage:	Room temp. for 6 hours
5 Cycle Freeze/Thaw:	5 cycles to -20°C
Standard Solution:	Not determined

This project was requested in a COR letter dated December 6, 1995. An interim report was submitted for review August 22, 1997 for a chloroquine/monodesethylchloroquine method for human blood. A December 8, 1997, fax from the COR requested changes in the interim report which were incorporated into final Study Report 29A dated August 11, 2000.

This report describes the analytical method and validation of the analytical method used to measure concentrations of chloroquine and monodesethylchloroquine in human blood samples. The method was developed and validated at the Analytical Division, Drug Studies Unit (DSU), UCSF, San Francisco, CA. The analysis of human blood samples was accomplished by use of the liquid chromatographic/mass spectrometric/mass spectrometric (LC/MS/MS) method.

Method Summary: Human blood samples (100 µl) were analyzed for chloroquine free base (CHL), and monodesethylchloroquine (MDC) with an LC/MS/MS procedure in a PE Sciex-API III® system that uses a silica gel column, an acetonitrile/water/TFA (90:10:0.1, v/v) with 5 mM ammonium acetate mobile phase, and mass spectrometric detection with sample inlet by heated nebulizer, positive ionization by APCI (Atmospheric Pressure Chemical Ionization) and mass scanning by MRM (Multiple Reaction Monitoring) analysis. The MS/MS analysis selectively monitors fragments referred to as parent ions. Since there may be several ions with the same molecular weight, these parent ions are further fragmented into daughter ions. Quantitation based on daughter ion response allows for greater selectivity and quantification of individual components. This greater selectivity allows for quanitification of components

that may be overlapping peaks with conventional HPLC. Sample cleanup consisted of addition of water and sonication to lyse cells, precipitation with a neostigmine internal standard solution in acetonitrile, centrifugation and transfer of the supernatant prior to separation by LC/MS/MS.

Validation Results Summary: The following validation parameters were evaluated for CHL and MDC.

1. Specificity: No significant endogenous interfering peaks for CHL, MDC, or for the internal standard were observed in blank human blood.

2. Inter-Day Precision and Accuracy: Interday precision and accuracy measurements were determined by analyzing quality control (QC) samples made of human blood spiked with known amounts of drug and metabolite. Each of 6 sets (n=2) of control samples at 4 different drug and metabolite concentrations was evaluated (6 standard curves for the drug and metabolite were run). Precision coefficients of variation (CV), ranged from 7.57% to 11.4% for CHL and from 8.51% to 13.0% for MDC. The accuracy, defined by the relative error (RE) ranged from -7.49% to -1.99% for CHL and from -8.28% to -4.87% for MDC.

3. Intra-Day Precision and Accuracy: Intraday precision and accuracy measurements were determined by analyzing quality control (QC) samples made of human blood spiked with known amounts of drug and metabolite. For intraday precision, 6 sets (n=1) of control samples for each of 4 different drug and metabolite concentrations were evaluated with 1 standard curve on the same run. CVs ranged from 2.04% to 8.22% for CHL and from 2.62% to 9.84% for MDC. R.E.s ranged from -8.08% to +0.210% for CHL and from -11.4% to +0.103% for MDC.

4. Lower Limit of Quantitation (LLOQ): The LLOQs for this assay are equivalent to the low points of the standard curves, or 20.0 ng/ml each for CHL and MDC. Interday mean, CV, and RE results are 16.9 ng/ml, 5.93%, and -15.4% for CHL and 17.8 ng/ml, 11.0%, and -10.8 for MDC. Intraday mean, CV, and RE results are 22.6 ng/ml, 9.81%, and +12.9% for CHL and 22.2 ng/ml, 8.34%, and +11.2% for MDC.

5. Linear Range: The validated linear concentration ranges for this assay were 20.0 to 2000 ng/ml for CHL and MDC.

6. Recovery: Peak area ratios of processed samples to unprocessed samples provided overall recoveries of 70.4% for CHL and 61.3% for MDC.

7. Stability:

    a. Freeze/Thaw: CHL and MDC were shown to be stable in human blood for up to 5 freeze/thaw cycles when samples are frozen to -20°C and thawed to room temperature.

    b. Bench Top: CHL and MDC were shown to be stable for at least 6 hours in human blood at ambient temperature.

c. Processed Samples: CHL, MDC, and internal standard were shown to be stable up to 27 days at 4°C. A test at the ambient temperature is yet to be performed.

d. Long Term: A test of stability of -20°C freezer storage is yet to be performed.

Human blood samples (100 µl) were analyzed for chloroquine and mono-desethyl-chloroquine with an LC/MS/MS procedure in a PE Sciex-API III system equipped with a silica column (4.6 x 50 mm, 5 µm particle size), 90% CH_3CN, 0.1% trifluoroacetic acid (TFA) and (final concentration) 5 mM ammonium acetate mobile phase and mass spectrometric detection with sample inlet by heated nebulizer, positive ionization by APCI (atmospheric pressure chemical ionization) and mass scanning by MRM (Multiple Reaction Monitoring) analysis. Sample preparation consisted of addition of 50 µl of water, sonication of the mixture, addition of neostigmine bromide internal standard (IS), and transfer of the supernatant prior to separation by LC/MS/MS. Standard curve and quality control (QC) samples were generated by spiking interference free human blood samples with known amounts of chloroquine, monodesethylchloroquine, and IS. Standard curve, QC and assay samples were prepared as described, then 1-2 µl aliquots were injected into the LC/MS/MS system for chromatographic separation and subsequent mass spectrometric detection. The peak area ratios of chloroquine and monodesethylchloroquine to IS were calculated for each sample from the measured peak areas obtained by LC/MS/MS. Finally, spiked concentrations and chloroquine and mono-desethyl-chloroquine to IS peak area ratios of the standard curve samples were fit by 1/y weighted least squares linear regression to the two equations for the best straight lines ($y = mx + b$, where y = peak area ratio and x = chloroquine or monodesethylchloroquine concentrations), and drug and metabolite concentrations in assay samples were calculated by these equations from the chloroquine and monodesethyl-chloroquine to IS peak area ratios obtained by LC/MS/MS.

Calibration standards and validation samples with drug concentrations within the calibration range of the assay were analyzed to assess the performance of the assay. Calibration standards and validation samples were generated by spiking blank human blood with chloroquine and monodesethylchloroquine. A didesethylchloroquine stock solution was also generated, diluted into working solutions and spiked into validation samples but data is not presented in this report, since validation acceptance criteria were not met. The didesethylchloroquine interday, intraday, and LOQ coefficients of variation and relative errors are not acceptable according to the SOP. Benchtop and Freeze/Thaw stabilities must be reanalyzed.

Conclusion: The LC/MS/MS method for analysis of human blood to determine concentrations of CHL and MDC was validated for the concentration ranges of 20.0 ng/ml to 2000 ng/ml for CHL and MDC. The method was demonstrated to be precise, accurate, and sufficiently reproducible for analysis of study samples.

Study Report 29B: Chloroquine, Monodesethylchloroquine and Didesethylchloroquine in Human Blood and Plasma

Study Characteristics: Study Report 29B

Test Article:	Chloroquine Monodesethylchloroquine Didesethylchloroquine
Test System:	human blood human plasma
Internal Standard:	Neostigmine bromide
Sample Assay Volume:	100 µl
Sample Cleanup:	Blood: Lyse cells with water Blood and plasma: precipitate proteins with acetonitrile

Analytical System

Detector:	MS/MS
Column Type:	Hypersil silica
Column Size:	4.6x50 mm, 3 µ particle size
Mobile Phase:	CH_3CN/H_2O (9:1, v/v) with 0.1% TFA and 5mM ammonium acetate

Validation Results: Chloroquine free base in human blood

Lower Limit of Quantitation:	20 ng/ml
Interday Mean, CV and RE:	20.0 ng/ml, 8.26%, and –0.250%
Intraday Mean, CV and RE:	16.7 ng/ml, 7.06%, and –16.7%
Standard curve range:	20 to 2000 ng/ml
Interday Precision Concentrations:	40, 500, and 1500 ng/ml
CV Range:	2.57% to 5.67%
RE Range:	–2.56% to +4.39%
Intraday Precision Concentrations:	40, 500, and 1500 ng/ml
CV Range:	1.50% to 5.53%
RE Range:	–4.29% to +7.78%
Blind Sample Assay	
Concentration Range:	Not determined
RE Range:	Not determined
Overall Mean Recovery:	63.1%
Stability	
Plasma Freezer Storage:	10 months at -70°C
Processed Sample:	8.5 hours at ambient temp. 4°C for 31 days
Plasma Storage:	6 hours at ambient temp.
5 Cycle Freeze/Thaw:	5 cycles to -70°C
Standard Solution:	Not determined

Validation Results: Monodesethylchloroquine free base in human blood

Lower Limit of Quantitation:	20 ng/ml
Interday Mean, CV and RE:	19.8 ng/ml, 9.88%, and –1.17%
Intraday Mean, CV and RE:	16.1 ng/ml, 8.09%, and –19.4%
Standard curve range:	20 to 2000 ng/ml
Interday Precision Concentrations:	40, 500, and 1500 ng/ml
CV Range:	2.97% to 4.92%
RE Range:	–6.22% to +2.22%
Intraday Precision Concentrations:	40, 500, and 1500 ng/ml
CV Range:	2.31% to 10.8%
RE Range:	-6.00% to +12.3%
Blind Sample Assay	
Concentration Range:	Not determined
RE Range:	Not determined
Overall Mean Recovery:	41.5%
Stability	
Plasma Freezer Storage:	10 months at -70°C
Processed Sample:	8.5 hours at ambient temp.
	4°C for 31 days
Plasma Storage:	6 hours at ambient temp.
5 Cycle Freeze/Thaw:	5 cycles to -70°C
Standard Solution:	Not determined

Validation Results: Didesethylchloroquine free base in human blood

Lower Limit of Quantitation:	20 ng/ml
Interday Mean, CV and RE:	21.6 ng/ml, 8.39%, and +7.75%
Intraday Mean, CV and RE:	17.9 ng/ml, 15.0%, and –10.67%
Standard curve range:	20 to 2000 ng/ml
Interday Precision Concentrations:	40, 500, and 1500 ng/ml
CV Range:	1.94% to 6.29%
RE Range:	-10.5% to +4.56%
Intraday Precision Concentrations:	40, 500, and 1500 ng/ml
CV Range:	2.26% to 7.43%
RE Range:	-11.0% to +13.0%
Blind Sample Assay	
Concentration Range:	Not determined
RE Range:	Not determined
Overall Mean Recovery:	54.6%
Stability	
Plasma Freezer Storage:	Not determined
Processed Sample:	8.5 hours at ambient temp.
	4°C for 31 days
Plasma Storage:	6 hours at ambient temp.
5 Cycle Freeze/Thaw:	5 cycles to -70°C
Standard Solution:	Not determined

Validation Results: Chloroquine free base in human plasma

Lower Limit of Quantitation:	20 ng/ml
Interday Mean, CV and RE:	17.6 ng/ml, 2.80%, and –11.8%
Intraday Mean, CV and RE:	Not determined
Standard curve range:	20 to 2000 ng/ml
Interday Precision Concentrations:	40, 100, 250, and 1500 ng/ml
CV Range:	3.60% to 8.12%
RE Range:	–2.33% to +10.1%
Intraday Precision Concentrations:	40, 100, 250, and 1500 ng/ml
CV Range:	1.28% to 6.68%
RE Range:	–11.3% to +4.00%
Blind Sample Assay	
Concentration Range:	Not determined
RE Range:	Not determined
Overall Mean Recovery:	88.1%
Stability	
Plasma Freezer Storage:	Not determined
Processed Sample:	Not determined
Plasma Storage:	Not determined
5 Cycle Freeze/Thaw:	Not determined
Standard Solution:	Not determined

Validation Results: Monodesethylchloroquine free base in human plasma

Lower Limit of Quantitation:	20 ng/ml
Interday Mean, CV and RE:	19.0 ng/ml, 3.80%, and –5.00%
Intraday Mean, CV and RE:	Not determined
Standard curve range:	20 to 2000 ng/ml
Interday Precision Concentrations:	40, 100, 250, and 1500 ng/ml
CV Range:	2.02% to 7.67%
RE Range:	–4.21% to +9.93%
Intraday Precision Concentrations:	40, 100, 250, and 1500 ng/ml
CV Range:	2.22% to 3.66%
RE Range:	-10.0% to +7.07%
Blind Sample Assay	
Concentration Range:	Not determined
RE Range:	Not determined
Overall Mean Recovery:	88.1%
Stability	
Plasma Freezer Storage:	Not determined
Processed Sample:	Not determined
Plasma Storage:	Not determined
5 Cycle Freeze/Thaw:	Not determined
Standard Solution:	Not determined

Validation Results: Didesethylchloroquine free base in human plasma

Lower Limit of Quantitation:	20 ng/ml
Interday Mean, CV and RE:	20.2 ng/ml, 10.8%, and +1.17%
Intraday Mean, CV and RE:	Not determined
Standard curve range:	20 to 2000 ng/ml
Interday Precision Concentrations:	40, 100, 250, and 1500 ng/ml
CV Range:	2.31% to 11.3%
RE Range:	-1.57% to +6.40%
Intraday Precision Concentrations:	40, 100, 250, and 1500 ng/ml
CV Range:	1.12% to 13.9%
RE Range:	-5.17% to +5.07%
Blind Sample Assay	
Concentration Range:	Not determined
RE Range:	Not determined
Overall Mean Recovery:	83.5%
Stability	
Plasma Freezer Storage:	Not determined
Processed Sample:	Not determined
Plasma Storage:	Not determined
5 Cycle Freeze/Thaw:	Not determined
Standard Solution:	Not determined

Study Description: Draft Study Report 29B on validation of a method for chloroquine, monodesethylchloroquine and didesethylchloroquine in human blood and including a short validation in human plasma was submitted for review September 23, 1999.

Human Blood

This report describes the analytical method and validation of the analytical method used to measure concentrations of chloroquine (and its metabolites) in human blood samples. The method was developed and validated at the Analytical Division, Drug Studies Unit (DSU), UCSF, San Francisco, CA. The method was requested by the Contracting Officer's Representative at the Walter Reed Army Institute of Research (WRAIR) for contract DAMD17-97-C-7058.

Method Summary: Human blood samples (100 µl) were analyzed for chloroquine (and its metabolites) with an LC/MS/MS procedure in a PE Sciex-API III® triple quadrupole system that uses a silica gel column, an acetonitrile/water/TFA (90:10:0.1, v/v) with 5 mM ammonium acetate mobile phase, and mass spectrometric detection with sample inlet by heated nebulizer, positive ionization by APCI (Atmospheric Pressure Chemical Ionization) and mass scanning by MRM (Multiple Reaction Monitoring) analysis. Sample cleanup consisted of addition of water, sonication, precipitation with a neostigmine internal standard solution in acetonitrile, centrifugation and transfer of the supernatant prior to separation by LC/MS/MS.

Validation Results Summary: The following validation parameters were evaluated for chloroquine (CHL) and its metabolites monodesethyl-chloroquine (MCL) and didesethylchloroquine (DCL).

1. Specificity: No significant endogenous interfering peaks for chloroquine (or its metabolites) or for the internal standard were observed in blank human blood.

2. Inter-Day Precision and Accuracy: Interday precision and accuracy measurements were determined by analyzing quality control (QC) samples made of human blood spiked with known amounts of drug. Each of 6 sets (n=2) of control samples at 3 different drug concentrations was evaluated (6 standard curves for the drug were run). Precision coefficients of variation (CV), ranged from 2.57% to 5.67% for CHL, 1.94% to 6.29% for DCL, and 2.97% to 4.92% for MCL. The accuracy, defined by the relative error (RE) ranged from –2.56% to +4.39% for CHL, -10.5% to +4.56% for DCL, and –6.22% to +2.22% for MCL.

3. Intra-Day Precision and Accuracy: Intraday precision and accuracy measurements were determined by analyzing quality control (QC) samples made of human blood spiked with known amounts of drug. For intraday precision, 6 sets (n=1) of control samples for each of three different drug concentrations were evaluated with one standard curve on the same run. CVs ranged from 1.50% to 5.53% for CHL, 2.26% to 7.43% for DCL, and 2.31% to 10.8% for MCL. The REs ranged from –4.29% to +7.78% for CHL, -11.0% to +13.0% for DCL, -6.00% to +12.3% for MCL.

4. Lower Limit of Quantitation (LLOQ): The LLOQs for this assay are equivalent to the low points of the standard curves, or 20.0 ng/ml for CHL, DCL and MCL. Interday mean, CV, and RE results are 20.0 ng/ml, 8.26%, and –0.250% for CHL, 21.6 ng/ml, 8.39%, and +7.75% for DCL, and 19.8 ng/ml, 9.88%, and –1.17% for MCL. Intraday mean, CV, and RE results are 16.7 ng/ml, 7.06%, and –16.7% for CHL, 17.9 ng/ml, 15.0%, and –10.6% for DCL, and 16.1 ng/ml, 8.09%, and –19.4% for MCL.

5. Linear Range: The validated linear concentration ranges for this assay were 20.0 to 2000 ng/ml for CHL, DCL, and MCL.

6. Recovery: Peak area ratios of processed samples to unprocessed samples provided overall recoveries of 63.1% for CHL, 41.5% for DCL, and 54.6% for MCL.

7. Stability:

   a. Freeze/Thaw: CHL, DCL, and MCL were shown to be stable in human blood for up to 5 freeze/thaw cycles when samples are frozen to -70°C and thawed to room temperature.

   b. Bench Top: CHL, DCL, and MCL were shown to be stable for at least 6 hours in human blood at ambient temperature.

c. Processed Sample – Ambient temperature: CHL, DCL, MCL, and internal standard were shown to be stable up to 8.5 hours at ambient temperature.

Processed Sample - Refrigerated: CHL, DCL, MCL, and internal standard were shown to be stable up to 31 days at 4°C.

d. Long Term: CHL and MCL were shown to be stable for up to 10 months in human blood at -70°C.

Human blood samples (100 µl) were analyzed for chloroquine, monodesethylchloroquine, and didesethylchloroquine with an LC/MS/MS procedure in a PE Sciex-API III system equipped with a silica column (4.6 x 50 mm, 3 µm particle size), 90% CH_3CN, 0.1% trifluoroacetic acid (TFA) and 5 mM ammonium acetate mobile phase and mass spectrometric detection with sample inlet by heated nebulizer, positive ionization by APCI (atmospheric pressure chemical ionization) and mass scanning by MRM (Multiple Reaction Monitoring) analysis. Sample preparation consisted of addition of 50 µl of water, sonication of the mixture for 5 minutes, addition of 400 µl of acetonitrile containing neostigmine internal standard (IS), mixing of the mixture for 1 minute, centrifugation for 10 minutes and transfer of the supernatant prior to separation by LC/MS/MS. Standard curve and quality control (QC) samples were generated by spiking interference free human blood samples with known amounts of chloroquine monodesethyl-chloroquine, didesethylchloroquine, and IS. Standard curve, QC and assay samples were prepared as described, then ~2 µl aliquots were injected into the LC/MS/MS system for chromatographic separation and subsequent mass spectrometric detection. The peak area ratios of chloroquine (daughter ion at 247 *m/z* from parent ion at 321 *m/z*) and monodesethylchloroquine (daughter ion at 114 *m/z* from parent ion at 292 *m/z*), didesethylchloroquine (daughter ion at 179 *m/z* from parent ion at 265 *m/z*) to IS (daughter ion at 72 *m/z* from parent ion at 209 *m/z*) were calculated for each sample from the measured peak areas obtained by LC/MS/MS. Finally, spiked concentrations of chloroquine, monodesethylchloroquine, and didesethylchloroquine to IS peak area ratios of the standard curve samples were fit by 1/y weighted least squares linear regression to the three equations for the best straight lines ($y = mx + b$, where y = peak area ratio and x = chloroquine, monodesethylchloroquine, or didesethylchloroquine concentrations), and drug and metabolite concentrations in assay samples were calculated by these equations from the chloroquine, monodesethylchloroquine, and didesethylchloroquine to IS peak area ratios obtained by LC/MS/MS.

Calibration standards and validation samples with drug concentrations within the calibration range of the assay were analyzed to assess the performance of the assay. Calibration standards and validation samples were generated by spiking blank human blood with chloroquine, monodesethyl-chloroquine, and didesethylchloroquine.

Retention times (approximate, in minutes) were internal standard 1:22, chloroquine 1:54, monodesethylchloroquine 1:40, didesethylchloroquine 1:40. The time between injections was 2-3 minutes.

Conclusion: The LC/MS/MS method for analysis of human blood to determine concentrations of chloroquine (and its metabolites) was validated for the concentration ranges of 20.0 ng/ml to 2000 ng/ml for chloroquine monodesethylchloroquine, and didesethylchloroquine. "Preparation time for a run of 40 sponsor samples plus standard curve and control samples is about 1&1/2 hours. Run time is about 3&1/4 hours." The method was demonstrated to be precise, accurate, and sufficiently reproducible for analysis of study samples.

Human Plasma

This report describes the analytical method and validation of the analytical method used to measure concentrations of chloroquine (and its metabolites) in human plasma samples. The method was developed and validated at the Analytical Division, Drug Studies Unit (DSU), UCSF, San Francisco, CA. The method was requested by the Contracting Officer's Representative at the Walter Reed Army Institute of Research (WRAIR) for contract DAMD17-97-C-7058.

Method Summary: Human plasma samples (100 µl) were analyzed for chloroquine (and its metabolites) with an LC/MS/MS procedure in a PE Sciex-API III® triple quadrupole system that uses a silica gel column, an acetonitrile/water/TFA (90:10:0.1, v/v) with 5 mM ammonium acetate mobile phase, and mass spectrometric detection with sample inlet by heated nebulizer, positive ionization by APCI (Atmospheric Pressure Chemical Ionization) and mass scanning by MRM (Multiple Reaction Monitoring) analysis. Sample cleanup consisted of precipitation with a neostigmine internal standard solution in acetonitrile, centrifugation and transfer of the supernatant prior to separation by LC/MS/MS.

Validation Results Summary: The following validation parameters were evaluated for chloroquine (CHL) and its metabolites monodesethyl-chloroquine (MCL) and didesethylchloroquine (DCL).

1. Specificity: No significant endogenous interfering peaks for chloroquine (or its metabolites) or for the internal standard were observed in blank human plasma.

2. Inter-Day Precision and Accuracy: Interday precision and accuracy measurements were determined by analyzing quality control (QC) samples made of human plasma spiked with known amounts of drug. Each of 3 sets (n=2) of control samples at 4 different drug concentrations was evaluated (3 standard curves for the drug were run). Precision coefficients of variation (CV), ranged from 3.60% to 8.12% for CHL, 2.31% to 11.3% for DCL, and 2.02% to 7.67% for MCL. The accuracy, defined by the relative error (RE) ranged from –2.33% to +10.1% for CHL, -1.57% to +6.40% for DCL, and –4.21% to +9.93% for MCL.

3. Intra-Day Precision and Accuracy: Intraday precision and accuracy measurements were determined by analyzing quality control (QC) samples made of human plasma spiked with known amounts of drug. For intraday precision, 6 sets (n=1) of control samples for each of four different drug concentrations were evaluated with one standard curve on the same run. CVs ranged from 1.28% to 6.68% for CHL, 1.12% to 13.9% for DCL, and 2.22% to 3.66% for MCL. The REs ranged from –11.3% to +4.00% for CHL, -5.17% to +5.07% for DCL, -10.0% to +7.07% for MCL.

4. Lower Limit of Quantitation (LLOQ): The LLOQs for this assay are equivalent to the low points of the standard curves, or 20.0 ng/ml for CHL, and DCL. Interday mean, CV, and RE results are 17.6 ng/ml, 2.80%, and –11.8% for CHL, 20.2 ng/ml, 10.8%, and +1.17% for DCL, and 19.0 ng/ml, 3.80%, and –5.00% for MCL.

5. Linear Range: The validated linear concentration ranges for this assay were 20.0 to 2000 ng/ml for CHL, DCL, and MCL.

6. Recovery: Peak area ratios of processed samples to unprocessed samples provided overall recoveries of 88.1% for CHL, 83.5% for DCL, and 88.1% for MCL.

Human plasma samples (100 µl) were analyzed for chloroquine, mono-desethylchloroquine, and didesethylchloroquine with an LC/MS/MS procedure in a PE Sciex-API III system equipped with a silica column (4.6 x 50 mm, 3 µm particle size), 90% CH_3CN, 0.1% trifluoroacetic acid (TFA) and 5 mM ammonium acetate mobile phase and mass spectrometric detection with sample inlet by heated nebulizer, positive ionization by APCI (atmospheric pressure chemical ionization) and mass scanning by MRM (Multiple Reaction Monitoring) analysis. Sample preparation consisted of addition of 400 µl of acetonitrile containing neostigmine internal standard (IS), mixing of the mixture for 1 minute, centrifugation for 5 minutes and transfer of the supernatant prior to separation by LC/MS/MS. Standard curve and quality control (QC) samples were generated by spiking interference free human plasma samples with known amounts of chloroquine, monodesethyl-chloroquine, didesethylchloroquine, and IS. Standard curve, QC and assay samples were prepared as described, then ~2 µl aliquots were injected into the LC/MS/MS system for chromatographic separation and subsequent mass spectrometric detection. The peak area ratios of chloroquine (daughter ion at 247 *m/z* from parent ion at 321 *m/z*) and monodesethylchloroquine (daughter ion at 114 *m/z* from parent ion at 292 *m/z*), didesethylchloroquine (daughter ion at 179 *m/z* from parent ion at 265 *m/z*) to IS (daughter ion at 72 *m/z* from parent ion at 209 *m/z*) were calculated for each sample from the measured peak areas obtained by LC/MS/MS. Finally, spiked concentrations of chloroquine, monodesethylchloroquine, and didesethylchloroquine to IS peak area ratios of the standard curve samples were fit by 1/y weighted least squares linear regression to the three equations for the best straight lines ($y = mx + b$, where y = peak area ratio and x = chloroquine, monodesethyl-chloroquine, or didesethylchloroquine concentrations), and drug and metabolite concentrations in assay samples were calculated by these

equations from the chloroquine, monodesethylchloroquine, and didesethylchloroquine to IS peak area ratios obtained by LC/MS/MS.

Calibration standards and validation samples with drug concentrations within the calibration ranges of the assay were analyzed to assess the performance of the assay. Calibration standards and validation samples were generated by spiking blank human plasma with chloroquine, monodesethyl-chloroquine, and didesethylchloroquine.

Retention times (approximate, in minutes) were internal standard 1:23, chloroquine 1:57, monodesethylchloroquine 1:44, didesethylchloroquine 1:45. The time between injections was 2-3 minutes.

Conclusion: The LC/MS/MS method for analysis of human plasma to determine concentrations of chloroquine (and its metabolites) was validated for the concentration ranges of 20.0 ng/ml to 2000 ng/ml for chloroquine monodesethylchloroquine, and didesethylchloroquine. "Preparation time for a run of 40 sponsor samples plus standard curve and control samples is about 1&1/2 hours. Run time is about 3&1/4 hours." The method was demonstrated to be precise, accurate, and sufficiently reproducible for analysis of study samples.

Study Report 30: WR 243251 in Human Plasma

Study Characteristics: Study Report 30	
Test Article:	WR 243251
Test System:	human plasma
Analytical System	
Detector:	MS/MS

This project was requested in a COR letter dated May 6, 1996. WR 243,251 standard compound was received May 10, 1996. Method development is in progress.

Study Report 32: WR 238,608 in Human Plasma and Blood

Study Characteristics: Study Report 32

Test Article:	WR 238,608
Test System:	human plasma
Internal Standard:	Verapamil
Sample Assay Volume:	100 µl
Sample Cleanup:	acetonitrile precipitation

Analytical System

Detector:	MS/MS
Column Type:	hypersil silica
Column Size:	4.6x50 mm, 5µ particle size
Mobile Phase:	acetonitrile/water/TFA (90:10:0.06, v/v/v).

Validation of a method for WR 238,605 in human plasma and blood by LC/MS/MS has been completed and a report is in preparation. A long term stability study for WR 238,605 in human blood and plasma (plasma data to 21 months and blood to 7 months was Faxed to the COR on May 22, 1998) for up to two years requested in a COR letter dated Feb. 15, 1996 continued. Results on analyses of 35 blind human plasma samples received February 25, 1998 from WRAIR were Faxed to the COR on May 21, 1998.

Study Report 32: WR 238,608 in Human Blood Small Volume Assay

Study Characteristics: Study Report 32 Small Volume Assay

Test Article:	WR 238,608
Test System:	human blood
Internal Standard:	Verapamil
Sample Assay Volume:	50 µl
Sample Cleanup:	Incubate in methanol/water, sonicate and precipitate proteins with acetonitrile

Analytical System

Detector:	MS/MS
Column Type:	Hypersil silica
Column Size:	4.6x50 mm, 5 µ particle size
Mobile Phase:	CH_3CN/H_2O (9:1, v/v) with 0.06% TFA

Validation Results: WR 238,608 free base in human blood

Lower Limit of Quantitation:	5.00 ng/ml
Interday Mean, CV and RE:	5.40 ng/ml, 8.11%, and +7.90%
Intraday Mean, CV and RE:	5.76 ng/ml, 4.31%, and +15.1%
Standard curve range:	5.00 to 800 ng/ml
Interday Precision Concentrations:	10, 100, and 600 ng/ml
CV Range:	4.31% to 5.09%
RE Range:	−1.45% to +2.96%
Intraday Precision Concentrations:	10, 100, and 600 ng/ml
CV Range:	1.74% to 3.93%
RE Range:	-2.55% to +3.83%
Blind Sample Assay	
Concentration Range:	Not determined
RE Range:	Not determined
Overall Mean Recovery:	69.9%
Stability	
Blood Freezer Storage:	Not determined
Processed Sample:	Room Temp. for 3.5 hours 4°C for 28 days
Blood Storage:	Room temp. for 6 hours
5 Cycle Freeze/Thaw:	5 cycles to -70°C
Standard Solution:	Not determined

A small volume sample (50 µl blood) method described in Faxes from the COR dated April 13, 21 and 24, 1998 has been validated and a draft report was submitted for review on April 12, 2000.

Study Description: This report describes the analytical method and validation of the analytical method used to measure concentrations of WR 238605 in small volume human blood samples. The method was developed and validated at the Analytical Division, Drug Studies Unit (DSU), UCSF, San Francisco, CA. The method was requested by the Contracting Officer's Representative at the Walter Reed Army Institute of Research (WRAIR) for contract DAMD17-97-C-7058.

Method Summary: Human blood samples (50 µl) were analyzed for WR 238605 with an LC/MS/MS procedure in a PE Sciex-API III® triple quadrupole system that uses a silica column, an acetonitrile/water/TFA (90:10:0.06, v/v/v) mobile phase, and mass spectrometric detection with sample inlet by heated nebulizer, positive ionization by APCI (Atmospheric Pressure Chemical Ionization) and mass scanning by MRM (Multiple Reaction Monitoring) analysis. Sample cleanup consisted of addition of and incubation (30 minutes) in methanol and water, sonication for 5 minutes, precipitation with a verapamil internal standard solution in acetonitrile, centrifugation and transfer of the supernatant prior to separation by LC/MS/MS.

Validation Results Summary: The following validation parameters were evaluated for WR 238605.

1. Specificity: No significant (i.e., above the normal noise level) endogenous interfering peaks for WR 238605 or for the internal standard were observed in blank human blood.

2. Inter-Day Precision and Accuracy: Interday precision and accuracy measurements were determined by analyzing quality control (QC) samples made of human blood spiked with known amounts of drug. Each of 6 sets (n=2) of control samples at 3 different drug concentrations was evaluated (6 standard curves for the drug were run). Precision coefficients of variation (CV), ranged from 4.31% to 5.09% for WR 238605. The accuracy, defined by the relative error (RE) ranged from –1.45% to +2.96% for WR 238605.

3. Intra-Day Precision and Accuracy: Intraday precision and accuracy measurements were determined by analyzing quality control (QC) samples made of human blood spiked with known amounts of drug. For intraday precision, 6 sets (n=1) of control samples for each of 3 different drug concentrations were evaluated with 1 standard curve on the same run. CVs ranged from 1.74% to 3.93% for WR 238605. REs ranged from -2.55% to +3.83% for WR 238605.

4. Lower Limit of Quantitation (LLOQ): The LLOQ for this assay is equivalent to the low point of the standard curves, or 5.00 ng/ml for WR 238605. Interday mean, CV, and RE results are 5.40 ng/ml, 8.11%, and

+7.90% for WR 238605. Intraday mean, CV, and RE results are 5.76 ng/ml, 4.31%, and +15.1% for WR 238605.

5. Linear Range: The validated linear concentration range for this assay was 5.00 to 800 ng/ml for WR 238605. Precision standard curve CVs, ranged from 3.64% to 8.11% for WR 238605. REs ranged from –5.17% to +7.90% for WR 238605.

6. Recovery: Peak area ratios of processed samples to unprocessed samples provided an overall recovery of 69.9% for WR 238605.

7. Stability:

   a. Freeze/Thaw: WR 238605 was shown to be stable in human blood for up to 5 freeze/thaw cycles when samples are frozen to -70°C and thawed to room temperature.

   b. Bench Top: WR 238605 was shown to be stable for at least 6 hours in human blood at ambient temperature.

   c. Processed Sample – Ambient temperature: WR 238605 and internal standard were shown to be stable up to 3.5 hours at ambient temperature.

      Processed Sample - Refrigerated: WR 238605 and internal standard were shown to be stable up to 28 days at 4°C.

Method: Human blood samples (50 µl) were analyzed for WR 238605 with an LC/MS/MS procedure in a PE Sciex-API III system equipped with a silica column (4.6 x 50 mm, 5 µm particle size), CH_3CN, H_2O, 0.06% TFA (90:10:0.06, v/v/v) mobile phase and mass spectrometric detection with sample inlet by heated nebulizer, positive ionization by APCI (Atmospheric Pressure Chemical Ionization) and mass scanning by MRM (Multiple Reaction Monitoring) analysis. Sample preparation consisted of addition of and incubation (30 minutes) in 50 µl of methanol and 100 µl of water, sonication of the mixture for 5 minutes, precipitation with 300 µl of acetonitrile containing verapamil internal standard (IS), mixing of the solution for 1 minute, centrifugation for 10 minutes and transfer of the supernatant prior to separation by LC/MS/MS. Standard curve and quality control (QC) samples were generated by spiking interference free human blood samples with known amounts of WR 238605 and IS. Standard curve, QC and assay samples were prepared as described, then ~6 µl aliquots were injected into the LC/MS/MS system for chromatographic separation and subsequent mass spectrometric detection. The peak area ratios of WR 238605 (daughter at 86 *m/z* from parent ion at 464 *m/z*) to IS (daughter ion at 165 *m/z* from parent ion at 455 *m/z*) were calculated for each sample from the measured peak areas obtained by LC/MS/MS. Finally, spiked concentrations and WR 238605 to IS peak area ratios of the standard curve samples were fit by 1/y weighted least squares linear regression to the equation for the best straight line ($y = mx + b$, where y = peak area ratio and x = WR 238605 concentrations), and

drug concentrations in assay samples were calculated by this equation from the WR 238605 to IS peak area ratios obtained by LC/MS/MS.

Calibration standards and validation samples with drug concentrations within the calibration range of the assay were analyzed to assess the performance of the assay. Calibration standards and validation samples were generated by spiking blank human blood with WR 238605.

Retention times (approximate, in decimal minutes) were 1.43-internal standard and 2.15-WR 238605. The time between injections was 2-3 minutes.

Conclusion: The LC/MS/MS method for analysis of human blood to determine concentrations of WR 238,605 was validated for the concentration range of 5.00 to 800 ng/ml. The method was demonstrated to be precise, accurate, and sufficiently reproducible for analysis of study samples.

Study Report 33: Halofantrine and WR 178460 in Human Plasma and Blood

Study Characteristics: Study Report 33

Test Article:	Halofantrine WR 178460
Test System:	human plasma and blood
Analytical System	
Detector:	MS/MS

Long term (2 year) freezer stability study and LC/MS/MS method development is in progress for halofantrine and desbutylhalofantrine in human plasma and blood.

Study Report 34: WR 254421 in Human Plasma

Study Characteristics: Study Report 34	
Test Article:	WR 254421
Test System:	human plasma
Analytical System	
Detector:	MS/MS

This project was requested as described in a COR fax dated November 12, 1997 for quantitation of WR 254,421 (as Free Base) in human plasma. Assay development is in progress.

Study Report 35: Artelinic Acid in Dog Plasma

Study Characteristics: Study Report 35

Test Article:	Artelinic acid
Test System:	dog plasma
Internal Standard:	Indomethacin
Sample Assay Volume:	100 µl
Sample Cleanup:	Precipitate proteins with acetonitrile

Analytical System

Detector:	MS/MS
Column Type:	C8
Column Size:	4.6x50 mm, 3 µ particle size
Mobile Phase:	35% CH_3CN, 35% MeOH, 0.1% TFA

Validation Results: Artelinic acid in dog plasma

Lower Limit of Quantitation:	4 ng/ml
Interday Mean, CV and RE:	4.09 ng/ml, 11.4%, and +2.25%
Intraday Mean, CV and RE:	4.02 ng/ml, 18.7%, and +0.458%
Standard curve range:	4-4800 ng/ml
Interday Precision Concentrations:	8, 30, and 120 ng/ml
CV Low Range:	3.56% to 17.9%
RE Low Range:	+2.29% to +5.52%
Intraday Precision Concentrations:	8, 30, and 120 ng/ml
CV Low Range:	3.23% to 11.4%
RE Low Range:	-4.92% to +11.4%
Blind Sample Assay	
Concentration Range:	Not determined
RE Range:	Not determined
Overall Mean Recovery:	92.2%
Internal Standard Recovery Low Range	96.0%
Stability	
Plasma Freezer Storage:	3 months at -20°C
Processed Sample:	Room temp 5.5 hours 4°C for 14 days
Bench Top Plasma Storage:	Room temp. for 6 hours
5 Cycle Freeze/Thaw:	5 cycles to -20°C
Standard Solution:	Not determined

LC/MS/MS method development and validation was requested as described in a COR fax dated July 22, 1998. A final report was issued on March 29, 2000. Additional results for processed sample and long term stability will be presented as an amendment to the final report when available.

Study Description: This report describes the analytical method and validation of the analytical method used to measure concentrations of Artelinic Acid in dog plasma samples. The method was developed and validated at the Analytical Division, Drug Studies Unit (DSU), UCSF, San Francisco, CA. The method was requested by the Contracting Officer's Representative at the Walter Reed Army Institute of Research (WRAIR) for contract DAMD17-97-C-7058.

Method Summary: Dog plasma samples (100 µl) were analyzed for Artelinic Acid with an LC/MS/MS procedure in a PE Sciex-API III® triple quadrupole system that uses a C8 column, an acetonitrile/methanol/water/TFA (35:35:30:0.1, v/v) mobile phase, and mass spectrometric detection with sample inlet by heated nebulizer, positive ionization by APCI (Atmospheric Pressure Chemical Ionization) and mass scanning by MRM (Multiple Reaction Monitoring) analysis. Sample cleanup consisted of precipitation with a lovastatin internal standard solution in acetonitrile, centrifugation and transfer of the supernatant prior to separation by LC/MS/MS.

Validation Results Summary: The following validation parameters were evaluated for Artelinic Acid.

1. Specificity: No significant (i.e., above the normal noise level) endogenous interfering peaks for Artelinic Acid or for the internal standard were observed in blank dog plasma.

2. Inter-Day Precision and Accuracy: Interday precision and accuracy measurements were determined by analyzing quality control (QC) samples made of dog plasma spiked with known amounts of drug. Each of 6 sets (n=2) of control samples at 3 different drug concentrations was evaluated (6 standard curves for the drug were run). Precision coefficients of variation (CV), ranged from 7.69% to 8.86% for Artelinic Acid (High Range) and 6.90% to 7.96% for Artelinic Acid (Low Range). The accuracy, defined by the relative error (RE) ranged from –5.75% to -0.667% for Artelinic Acid (High Range) and +0.139% to +9.22% for Artelinic Acid (Low Range).

3. Intra-Day Precision and Accuracy: Intraday precision and accuracy measurements were determined by analyzing quality control (QC) samples made of dog plasma spiked with known amounts of drug. For intraday precision, 6 sets (n=1) of control samples for each of three different drug concentrations were evaluated with one standard curve on the same run. CVs ranged from 2.10% to 8.49% for Artelinic Acid (High Range) and 3.23% to 11.4% for Artelinic Acid (Low Range). The REs ranged from +4.13% to +5.48% for Artelinic Acid (High Range) and -4.92% to +11.4% for Artelinic Acid (Low Range).

4. Lower Limit of Quantitation (LLOQ): The LLOQ for this assay is equivalent to the low point of the low range standard curve, or 4.00 ng/ml for Artelinic Acid. In addition, the minimum limit of quantitation of the

high range curve is 60.0 ng/ml for Artelinic Acid. Interday mean, CV, and RE results are 4.09 ng/ml, 11.4%, and +2.25% for Artelinic Acid (Low Range) and 60.4 ng/ml, 5.79%, and +0.667% for Artelinic Acid (High Range). Intraday mean, CV, and RE results are 4.02 ng/ml, 18.7%, and +0.458% for Artelinic Acid (Low Range) and 63.8 ng/ml, 6.25%, and +6.28% for Artelinic Acid (High Range).

5. Linear Range: The validated linear concentration ranges for this assay were 60.0 to 4800 ng/ml for Artelinic Acid (High Range) 4.00 to 240 ng/ml Artelinic Acid (Low Range).

6. Recovery: Peak area ratios of processed samples to unprocessed samples provided an overall recovery of 92.2% for Artelinic Acid. Recovery averaged 110% (high range) and 103% (low range) for lovastatin (internal standard).

7. Stability:

   a. Freeze/Thaw: Artelinic Acid was shown to be stable in dog plasma for up to 5 freeze/thaw cycles when samples are frozen to -20°C and thawed to room temperature.

   b. Bench Top: Artelinic Acid was shown to be stable for at least 6 hours in dog plasma at ambient temperature.

   c. Processed Sample – Ambient temperature: Artelinic Acid and internal standard were shown to be stable up to 5.5 hours at ambient temperature.

      Processed Sample - Refrigerated: Artelinic Acid and internal standard were shown to be stable up to 3 days at 4°C, but internal standard (lovastatin) was shown to be not stable by 7 days at 4°C.

   d. Long Term: Artelinic Acid was shown to be stable for up to 3 months in dog plasma at -20°C.

Method: Dog plasma samples (100 µl) were analyzed for Artelinic Acid with an LC/MS/MS procedure in a PE Sciex-API III system equipped with a C8 column (4.6 x 50 mm, 3 µm particle size), 35% CH_3CN, 35% MeOH, 0.1% TFA mobile phase and mass spectrometric detection with sample inlet by heated nebulizer, positive ionization by APCI (atmospheric pressure chemical ionization) and mass scanning by MRM (Multiple Reaction Monitoring) analysis. Sample preparation consisted of addition of 200 µl of acetonitrile containing lovastatin internal standard (IS), mixing of the mixture for 1 minute, centrifugation for 5 minutes and transfer of the supernatant to an autosampler injection vial prior to separation by LC/MS/MS. Standard curve and quality control (QC) samples were generated by spiking interference free dog plasma samples with known amounts of Artelinic Acid and IS. Standard curve, QC and assay samples were prepared as described, then ~40 µl aliquots were injected into the LC/MS/MS

system for chromatographic separation and subsequent mass spectrometric detection. The peak area ratios of Artelinic Acid (high range daughter ion at 220 *m/z* and low range daughter at 162 *m/z* from parent ion at 373 *m/z*) to IS (daughter ion at 199 *m/z* from parent ion at 405 *m/z*) were calculated for each sample from the measured peak areas obtained by LC/MS/MS. Finally, spiked concentrations and (Artelinic Acid to IS) peak area ratios of the standard curve samples were fit by 1/y weighted least squares linear regression to the equations for the best straight lines (y = mx + b, where y = peak area ratio and x = Artelinic Acid concentrations), and drug concentrations in assay samples were calculated by these equations from the Artelinic Acid to IS peak area ratios obtained by LC/MS/MS.

Calibration standards and validation samples with drug concentrations within the calibration range of the assay were analyzed to assess the performance of the assay. Calibration standards and validation samples were generated by spiking blank dog plasma with Artelinic Acid.

Retention times (approximate, in decimal minutes) were internal standard 3.15 and Artelinic Acid 2.60. The time between injections was 4-5 minutes.

Conclusion: The LC/MS/MS method for analysis of dog plasma to determine concentrations of Artelinic Acid was validated for the concentration ranges of 4.00 ng/ml to 4800 ng/ml. The method was demonstrated to be precise, accurate, and sufficiently reproducible for analysis of study samples.

New Internal Standard Validation

Method Summary: Dog plasma samples (100 µl) were analyzed as described in the main body of this report, except that an indomethacin internal standard was used instead of a lovastatin internal standard.

Validation Results Summary: The following validation parameters (presented in correspondence with the main body of this report) were evaluated for Artelinic Acid with an indomethacin internal standard.

1. Specificity: No significant (i.e., above the normal noise level) endogenous interfering peaks for Artelinic Acid or for the internal standard were observed in blank dog plasma.

2. Inter-Day Precision and Accuracy: Interday precision and accuracy measurements were determined by analyzing quality control (QC) samples made of dog plasma spiked with known amounts of drug. Each of 6 sets (n=2) of control samples at 3 different drug concentrations was evaluated (6 standard curves for the drug were run). Precision coefficients of variation (CV), ranged from 3.17% to 3.79% for Artelinic Acid (High Range) and 3.56% to 17.9% for Artelinic Acid (Low Range). The accuracy, defined by the relative error (RE) ranged from –1.27% to +2.01% for Artelinic Acid (High Range) and +2.29% to +5.52% for Artelinic Acid (Low Range).

6. Recovery averaged 106% (high range) and 96.0% (low range) for Indomethacin (internal standard).

7. Stability:

   c. Processed Sample - Refrigerated: Artelinic Acid and internal standard were shown to be stable up to 14 days at 4°C.

Analytical Method

Dog plasma samples (100 µl) were analyzed for Artelinic Acid with an LC/MS/MS procedure as described in this report, except that an indomethacin internal standard was used instead of a lovastatin internal standard.

Sample preparation consisted of addition of 200 µl of acetonitrile containing indomethacin internal standard (IS). The peak area ratios of Artelinic Acid to IS (daughter ion at 139 *m/z* from parent ion at 359 *m/z*) were calculated for each sample from the measured peak areas obtained by LC/MS/MS.

Retention times (approximate, in decimal minutes) were internal standard 1.47 and Artelinic Acid 2.50. The time between injections was 4-5 minutes.

Study Report 35, Supplement I: Artelinic Acid in Human Plasma

Study Characteristics: Study Report 35, Supplement I

Test Article:	Artelinic acid
Test System:	human plasma
Internal Standard:	Indomethacin
Sample Assay Volume:	100 µl
Sample Cleanup:	Precipitate proteins with acetonitrile

Analytical System

Detector:	MS/MS
Column Type:	C8
Column Size:	4.6x50 mm, 3 µ particle size
Mobile Phase:	35% CH_3CN, 35% MeOH, 0.1% TFA

Validation Results: Artelinic acid in human plasma

Lower Limit of Quantitation:	4 ng/ml
Interday Mean, CV and RE:	4.05 ng/ml, 9.57%, and +1.33%
Intraday Mean, CV and RE:	3.87 ng/ml, 8.57%, and -3.15%
Standard curve range:	4-4800 ng/ml
Interday Precision Concentrations:	8, 30, and 120 ng/ml
CV Low Range:	4.06% to 9.91%
RE Low Range:	-2.29% to +4.98%
Intraday Precision Concentrations:	8, 30, and 120 ng/ml
CV Low Range:	3.57% to 12.2%
RE Low Range:	-1.67% to +13.2%
Overall Mean Recovery:	99.1%
Internal Standard Recovery Low Range	94.7%
Stability	
Plasma Freezer Storage:	3 months at -20°C
Processed Sample:	Room temp 7 hours 4°C for 14 days
Bench Top Plasma Storage:	Room temp. for 6 hours
5 Cycle Freeze/Thaw:	5 cycles to -20°C
Standard Solution:	Not determined

Study Description: Validation of a LC/MS/MS Method for the Determination of Artelinic Acid in Human Plasma Samples

This report describes the analytical method and validation of the analytical method used to measure concentrations of Artelinic Acid in human plasma samples. The method was developed and validated at the Analytical Division, Drug Studies Unit (DSU), UCSF, San Francisco, CA. The method was requested

by the Contracting Officer's Representative at the Walter Reed Army Institute of Research (WRAIR) for contract DAMD17-97-C-7058.
Method Summary: Human plasma samples (100 µl) were analyzed for Artelinic Acid with an LC/MS/MS procedure in a PE Sciex-API III® triple quadrupole system that uses a C8 column, an acetonitrile/methanol/water /TFA (35:35:30:0.1, v/v) mobile phase, and mass spectrometric detection with sample inlet by heated nebulizer, positive ionization by APCI (Atmospheric Pressure Chemical Ionization) and mass scanning by MRM (Multiple Reaction Monitoring) analysis. Sample cleanup consisted of precipitation with a indomethacin internal standard solution in acetonitrile, centrifugation and transfer of the supernatant prior to separation by LC/MS/MS.

Validation Results Summary: The following validation parameters were evaluated for Artelinic Acid.

1. Specificity: No significant (i.e., above the normal noise level) endogenous interfering peaks for Artelinic Acid or for the internal standard were observed in blank human plasma.

2. Inter-Day Precision and Accuracy: Interday precision and accuracy measurements were determined by analyzing quality control (QC) samples made of human plasma spiked with known amounts of drug. Each of 6 sets (n=2) of control samples at 3 different drug concentrations was evaluated (6 standard curves for the drug were run). Precision coefficients of variation (CV), ranged from 5.59% to 6.77% for Artelinic Acid (High Range) and 4.06% to 9.91% for Artelinic Acid (Low Range). The accuracy, defined by the relative error (RE) ranged from –4.58% to +1.28% for Artelinic Acid (High Range) and -2.29% to +4.98% for Artelinic Acid (Low Range).

3. Intra-Day Precision and Accuracy: Intraday precision and accuracy measurements were determined by analyzing quality control (QC) samples made of human plasma spiked with known amounts of drug. For intraday precision, 6 sets (n=1) of control samples for each of three different drug concentrations were evaluated with one standard curve on the same run. CVs ranged from 4.63% to 9.16% for Artelinic Acid (High Range) and 3.57% to 12.2% for Artelinic Acid (Low Range). The REs ranged from -5.14% to -0.771% for Artelinic Acid (High Range) and -1.67% to +13.2% for Artelinic Acid (Low Range).

4. Lower Limit of Quantitation (LLOQ): The LLOQ for this assay is equivalent to the low point of the low range standard curve, or 4.00 ng/ml for Artelinic Acid. In addition, the minimum limit of quantitation of the high range curve is 60.0 ng/ml for Artelinic Acid. Interday mean, CV, and RE results are 4.05 ng/ml, 9.57%, and +1.33% for Artelinic Acid (Low Range) and 55.8 ng/ml, 6.27%, and -6.97% for Artelinic Acid (High Range). Intraday mean, CV, and RE results are 3.87 ng/ml, 8.57%, and -3.15% for Artelinic Acid (Low Range) and 53.6 ng/ml, 6.02%, and -10.6% for Artelinic Acid (High Range).

5. Linear Range: The validated linear concentration ranges for this assay were 60.0 to 4800 ng/ml for Artelinic Acid (High Range) 4.00 to 240 ng/ml Artelinic Acid (Low Range).

6. Recovery: Peak area ratios of processed samples to unprocessed samples provided an overall recovery of 99.1% for Artelinic Acid. Recovery averaged 92.9% (high range) and 94.7% (low range) for indomethacin (internal standard).

7. Stability:

   a. Freeze/Thaw: Artelinic Acid was shown to be stable in human plasma for up to 5 freeze/thaw cycles when samples are frozen to -20°C and thawed to room temperature.

   b. Bench Top: Artelinic Acid was shown to be stable for at least 6 hours in human plasma at ambient temperature.

   c. Processed Sample – Ambient temperature: Artelinic Acid and internal standard were shown to be stable up to 7 hours at ambient temperature.

      Processed Sample - Refrigerated: Artelinic Acid and internal standard were shown to be stable up to 14 days at 4°C.

   d. Long Term: Artelinic Acid was shown to be stable for up to 3 months in human plasma at -20°C.

8. Matrix Effect: Artelinic Acid was shown to be unaffected by a matrix effect for 6 sources of human plasma.

9. Anticoagulant Interference: Analysis of quality control samples at three concentrations within the standard curve range, in duplicate, with a standard curve generated with plasma containing CPD as anticoagulant indicated acceptable control and standard curve back calculated results.

Analytical Method

Human plasma samples (100 µl) were analyzed for Artelinic Acid with an LC/MS/MS procedure in a PE Sciex-API III system equipped with a C8 column (4.6 x 50 mm, 3 µm particle size), 35% CH_3CN, 35% MeOH, 0.1% TFA mobile phase and mass spectrometric detection with sample inlet by heated nebulizer, positive ionization by APCI (atmospheric pressure chemical ionization) and mass scanning by MRM (Multiple Reaction Monitoring) analysis. Sample preparation consisted of addition of 200 µl of acetonitrile containing indomethacin internal standard (IS), mixing of the mixture for 1 minute, centrifugation for 5 minutes and transfer of the supernatant to an autosampler injection vial prior to separation by LC/MS/MS. Standard curve and quality control (QC) samples were generated by spiking interference free human plasma

samples with known amounts of Artelinic Acid and IS. Standard curve, QC and assay samples were prepared as described, then ~40 µl aliquots were injected into the LC/MS/MS system for chromatographic separation and subsequent mass spectrometric detection. The peak area ratios of Artelinic Acid (high range daughter ion at 220 *m/z* and low range daughter at 162 *m/z* from parent ion at 373 *m/z*) to IS (daughter ion at 139 *m/z* from parent ion at 359 *m/z*) were calculated for each sample from the measured peak areas obtained by LC/MS/MS. Finally, spiked concentrations and (Artelinic Acid to IS) peak area ratios of the standard curve samples were fit by 1/y weighted least squares linear regression to the equations for the best straight lines (y = mx + b, where y = peak area ratio and x = Artelinic Acid concentrations), and drug concentrations in assay samples were calculated by these equations from the Artelinic Acid to IS peak area ratios obtained by LC/MS/MS.

Calibration standards and validation samples with drug concentrations within the calibration range of the assay were analyzed to assess the performance of the assay. Calibration standards and validation samples were generated by spiking blank human plasma with Artelinic Acid.

Retention times (approximate, in decimal minutes) were internal standard 1.20 and Artelinic Acid 1.90. The time between injections was 4-5 minutes.

Conclusion

The LC/MS/MS method for analysis of human plasma to determine concentrations of Artelinic Acid was validated for the concentration range of 4.00 ng/ml to 4800 ng/ml for Artelinic Acid. "Preparation time for a run of 40 sponsor samples plus standard curve and control samples is about 3 hours. Run time is about 5 hours." The method was demonstrated to be precise, accurate, and sufficiently reproducible for analysis of study samples.

Study Report 35B: Artelinic Acid in Rat Plasma

Study Characteristics: Study Report 35	
Test Article:	Artelinic acid
Test System:	rat plasma
Analytical System	
Detector:	MS/MS

LC/MS/MS method development is in progress for artelinic acid in rat plasma. This project was described in a COR fax dated July 22, 1998.

Study Report 36: Gentamicin and Paromomycin in Human Urine

Study Characteristics: Study Report 36

Test Article:	Gentamicin Paromomycin
Test System:	human urine
Analytical System	
Detector:	UV

HPLC method development is in progress for gentamicin and paromomycin in human urine. This project was described in a COR fax dated January 31, 2000.

Routine Assay Results

The following section presents short descriptions of specific routine sample assays completed or currently in progress during the contract. Complete annual data findings are presented in Appendix B.

TABLE 5: CURRENT ROUTINE ANALYSES

Routine Analysis Report Title	Report Date	Test Article	Test System	No. of Samples	Report No.
For Halofantrine and WR 178, 460 (as fb) of Plasma Samples Obtained for Protocol Titled "Pharmacokinetics of a New Multiple Dose Halofantrine Regimen"	12/10/96 draft in review by COR	Halofantrine WR 178,460	human plasma	642 642	Hal/P 93-2
No protocol	2/25/94 data, draft in prep.	*p*-aminohept-anophenone	dog plasma	876	Pah/P 93-3
For Halofantrine and WR 178,460 (as f.b.) of Rat Liver, Bile and Perfusate Samples	10/28/94 final data, draft in prep	halofantrine	rat liver perfsate bile	no count	Hal/lpb 93-7
For WR 238,605 (as fb) Human Plasma and Blood Samples Obtained for the Protocol Titled "Pharmacokinetics, Pharmacodynamics, Safety and Tolerance of a Single Oral Dose of WR 238605 Succinate"	2/7/95 final data, draft in prep	WR 238,605	human plasma blood	359 359	WR5/PB 93-8
For *p*-Aminoheptanophenone of Dog Plasma Samples Obtained for Protocol Titled "p-Aminoheptanophenone (PAHP) (WR269410) Single Dose IV and Oral Pharmacokinetic, Pharmacodynamic, Bioavailability and Metabolism Study in Dogs"	2/7/95 final data, draft in prep	*p*-aminohept-anophenone	dog plasma	189	Pah/P 93-9
For WR 238,605 (as free base)Monkey Plasma Samples	11/22/94 finl data, let rp in prep	WR 238,605	monkey plasma	12	WR5/P 94-1
For PAHP Rat Plasma Samples Obtained for the Protocol Titled "p-Aminoheptanophenone (PAHP) (WR269410) Single Dose IV and Oral Pharmacokinetic, Pharmacodynamic, Bioavailabiity and Metabolism Study in Rats"	2/7/95 - final data, draft in prep	*p*-aminohept-anophenone	rat plasma	152	Pah/P 94-2

TABLE 5: CURRENT ROUTINE ANALYSES

Routine Analysis Report Title	Report Date	Test Article	Test System	No. of Samples	Report No.
Tentative Title: For WR 6026 and Metabolites in Plasma and Urine Samples Obtained for the Protocol Titled "Clinical Trial of Oral WR6026•2HCl in Patients with Brazilian Visceral Leishmaniasis due to L. chagasi: Initial Dose Range Determine	8/22/97 final data final data in progress final data final data final data	 WR 6026 WR 211789 WR 254421 WR 6026 WR 211789 WR 254421	human plasma urine 	 120 90 	WR6/PU 94-3
Tentative Title: For WR 238605 in Plasma Samples Obtained for the Protocol Titled "Evaluation of WR 238605 as a Prophylactic Agent against Induced P. falciparum Malaria Infection in Healthy Non-immune Subjects: A Dose Ranging Study"	11/21/94 final data, draft in prep	WR 238,605	human plasma blood	 28 28	WR5/PB 94-4
For WR 238605 in Plasma Samples Obtained for the Protocol Titled "WR 238605 Multiple Drug Interaction Study in Beagle Dogs"	6/11/99 draft report in review by COR	WR 238605 Mefloquine Chloroquine Quinine Doxycycline Halofantrine	human plasma	 1084	WR5/P 95-3
Routine Analysis for Halofantrine and WR 178460 in Plasma Samples Obtained for the Protocol Titled "Halofantrine as Prophylaxis against Malaria: Multiple-Dose Safety, Tolerance and Pharmacokinetics Study"	11/24/97 final data 5/4/98 final data draft in prep	 Halofantrine WR 178,460	human plasma	 1365 1365	Hal/P 95-4
HPLC Analysis for Determination of Enantiomers of Halofantrine and Metabolite in Human Plasma Samples Obtained for Protocol Titled "Halofantrine as Prophylaxis against Malaria: Multiple-dose Safety, Tolerance and Pharmacokinetics Study"	3/14/01 draft report in review by COR	 +Halofantrine -Halofantrine +WR 178,460 -WR 178,460	human plasma	 988 988 988 988	Hal/P 95-4 (Chiral)
Tentative Title: For WR 238605 in Plasma Samples Obtained for Protocol Titled "Dose-Ranging Study of the Safety and Efficacy of WR 238605 in the Prevention of Relapse of Plasmodium vivax Infection in Thailand"	3/2/98 final data draft in prep	WR238605	human plasma blood plasma blood	 558 552 558 552	WR5/BP 96-2

TABLE 5: CURRENT ROUTINE ANALYSES

Routine Analysis Report Title	Report Date	Test Article	Test System	No. of Samples	Report No.
LC/MS/MS Analysis for the Determination of Chloroquine, Didesethylchloroquine and Monodesethylchloroquine in Human Plasma and Blood Samples Obtained under the Protocol dated March 26, 1996 for IND Number 38503, Surgeon General, U.S. Army, Study No. 6 and Titled "Dose-Ranging Study of the Safety and Efficacy of WR 238605 in the Prevention of Relapse of *Plasmodium vivax* Infection in Thailand"	8/2/01 draft report in review	Chloroquine (C) DidesethylC Monodesethyl C	human plasma blood plasma blood plasma blood	 357 358 357 358 357 358	WR5/BP 96-2 (Chlor)
For WR238605 in Dog Plasma Samples for the Protocol Titled "One Year Oral Toxicity Study of WR 238605"	1/5/99 draft in revision	WR238605	dog plasma	224	WR5/P 97-1
For Mefloquine Chloroquine and Primaquine in Plasma Samples	12/11/97 final data, draft in prep	Mefloquine Chloroquine Primaquine	plasma	14 2 2	MEF/P 97-2
LC/MS/MS Analysis for the Determination of Artelinic Acid in Dog Plasma Samples Obtained under Protocol 7869.14.01 (Task Order SR99-1) Titled "Effect of Artelinic Acid on Dogs after Oral Administration for 14 Days"	4/14/00 final report	Artelinic acid	dog plasma	244	Art/dp 99-1
HPLC Analysis for the Determination of Paromomycin and Gentamicin in Human Plasma Samples Obtained under the Protocol (Version 3.1, IND 50,098, Log No. A-8225) Titled "Topical Treatment of Cutaneous Leishmaniasis with WR 279396: A Phase 2 Study in Colombia, Log No. A-8225 -- Version 3.1 -- 23 April 1998"	8/16/00 draft report in review	Gentamicin Paromomycin	human plasma	230	Gen/P 99-2

TABLE 5: CURRENT ROUTINE ANALYSES

Routine Analysis Report Title	Report Date	Test Article	Test System	No. of Samples	Report No.
HPLC Analysis for Determination of Paromomycin & Gentamicin in Human Urine Samples Obtained for Protocol (Version 3.1, IND 50,098, Log No. A-8225) Titled "Topical Treatment of Cutaneous Leishmaniasis with WR 279396: Phase 2 Study in Colombia, Log No. A-8225-Version 3.1-23 Apr 98"	in progress	Gentamicin Paromomycin	human urine	276	Gen/U 99-2
Inter-laboratory Validation Samples for Artelinic Acid Measurement	final data submitted 1/19/01	Artelinic acid	human plasma	10	Art/HP 00-1
Tentative Title: For Artelinic Acid in Dog Plasma Samples Obtained for Protocol Titled "Effect of Artelinic Acid on Dogs after Oral Administration for 14 Days"		Artelinic acid	dog plasma	Not yet rec'd	Art/HP 00-2
Tentative Title: For Artelinic Acid in Rat Plasma Samples Obtained for Protocol Titled "Effect of Artelinic Acid on Rats after Oral Administration for 14 Days"		Artelinic acid	rat plasma	Not yet rec'd	Art/HP 00-3

p-Aminoheptanophenone (WR 269,410), WR 258,948 and WR 302

Pah/P 93-3
(analytical data was presented in the DAMD17-92-C-2028 mid-term report)

Results will be reported in Analysis Report No. 93-3. Status of samples received is described in the table below. Report completion requires completion of method validation.

No. of Samples	Description	Date Received	Status
106	dog plasma	3/3/93	Results Faxed to COR 9/23/93
52	dog blood	3/3/93	Not to be assayed
645	dog plasma	9/21/93	Results Faxed to COR 2/25/94
36	blind spiked dog plasma	9/30/93 11/2/93	Results Faxed to COR 1/25/94
125	dog plasma	10/21/93	Results Faxed to COR 2/25/94

Pah/P 93-9
(analytical data was presented in the DAMD17-92-C-2028 final report)

Samples (189 dog plasma) were received July 12, 1994 to be analyzed in accordance with the protocol titled "*p*-Aminoheptanophenone (PAHP) (WR269410) Single Dose IV and Oral Pharmacokinetic, Pharmacodynamic, Bioavailability and Metabolism Study in Dogs." Analysis is complete and final results were Faxed to the COR on February 7, 1995. Report completion requires completion of the method validation report.

Pah/P 94-2
(analytical data was presented in the DAMD17-92-C-2028 final report)

Samples (152 rat plasma) were received July 12, 1994 to be analyzed in accordance with the protocol titled "*p*-Aminoheptanophenone (PAHP) (WR269410) Single Dose IV and Oral Pharmacokinetic, Pharmacodynamic, Bioavailability and Metabolism Study in Rats." Analysis is complete and final results were Faxed to the COR on February 7, 1995. Report completion requires completion of the method validation report.

ARTELINIC ACID

Art/P 00-1

Inter-laboratory validation samples for artelinic acid measurement generated in Thailand were analyzed and results were submitted January 19, 2001.

GENTAMICIN AND PAROMOMYCIN

Gen/PU 99-2
(analytical data is presented in Appendix B)

Samples were received (230 human plasma and 276 human urine) February 1, 2000 in accordance with the protocol titled "Topical Treatment of Cutaneous Leishmaniasis with WR 279396: A Phase 2 Study in Colombia." The plasma assay has been completed, final data was faxed to the COR August 15, 2000 and draft report Gen/P 99-2 was submitted for review August 16, 2000.

HALOFANTRINE

Hal/P 93-2
(analytical data was presented in the DAMD17-92-C-2028 final report)

Analysis of 642 human plasma samples for determination of the free base concentrations of halofantrine (WR 171,669) and of its metabolite (WR 178,460) was accomplished by use of an HPLC method described in Study Report 17, developed under contract DAMD17-86-C-6150. The samples were obtained from the South Florida Drug Research Corporation, Inc., in accordance with the protocol titled "Pharmacokinetics of a New Multiple Dose Halofantrine Regimen." Analytical results were presented in Analysis Report Hal/P 93-2

submitted for review on December 10, 1996, for plasma samples from human male subjects from analyses performed from April 30 through June 8, 1993.

Hal/lpb 93-7
(analytical data was presented in the DAMD17-92-C-2028 final report)

Final bile, liver and perfusate results were attached to Quarterly Report 11. Additional data, showing just perfusate extraction results, were Faxed December 28, 1994. Analysis Report Hal/Lprb 93-7 is in preparation. Remaining samples were returned to WRAIR on July 25, 1995.

Hal/P 95-4

Samples are to be analyzed in accordance to the protocol titled "Halofantrine as Prophylaxis against Malaria: Multiple-Dose Safety, Tolerance and Pharmacokinetics Study." Final data on 1060 samples for halofantrine and WR 178,460 (free base) concentrations were Faxed to the COR on January 3, 1997. Final results on 305 samples received on June 10, 1997 were Faxed to the COR on November 24, 1997. Analysis Report Hal/BP 95-4 is in preparation.

Hal/P 95-4 (Chiral)

Chiral results on the samples described above for Hal/P 95-4 were Faxed to the COR on May 4, 1998. Draft Analysis Report Hal/BP 95-4 (Chiral) was submitted for review on March 14, 2001.

MEFLOQUINE

Mef/P 97-2

Analysis of 14 human plasma samples for determination of the free base concentration of mefloquine hydrochloride (WR 142,490), was accomplished by use of the HPLC method described in Study Report 14B dated August 29, 1989 under contract DAMD17-86-C-6150. Final mefloquine concentrations on 14 of 15 human plasma samples (15th=NS) were submitted in a fax dated November 13, 1997. Final primaquine, chloroquine and metabolite concentrations on 2 human plasma samples were submitted in a fax dated December 11, 1997. The samples were obtained from the Division of Experimental Therapeutics, Walter Reed Army Institute of Research. A report is in preparation.

WR 238,605

WR5/P 93-8
(analytical data was presented in the DAMD17-92-C-2028 final report)

Routine analysis of 359 human plasma and 359 human blood samples was completed for Analysis Report WR5/BP 93-8 for samples received in accordance with the protocol titled "Pharmacokinetics, Pharmacodynamics, Safety and Tolerance of a Single Oral Dose of WR 238605 Succinate." Final data was attached to Quarterly Report 11. Repeat analysis of selected samples, as

requested in a FAX from the COR dated December 9, 1994, was completed and results were faxed to the COR February 7, 1995. A report is in preparation.

WR5/P 94-1
(analytical data was presented in the DAMD17-92-C-2028 final report)

Monkey blood (12) samples were received September 15, 1994. Final analytical results were faxed to the COR on 11/22/94. A brief letter reporting results and referring to the human validation report as suggested by the COR at the April 3, 1995 site visit is in preparation. The analysis was set to proceed with use of blank human plasma for standard curve and control samples and blank monkey plasma as duplicate controls.

WR5/P 94-4
(analytical data was presented in the DAMD17-92-C-2028 final report)

Human plasma (28) and human blood (28) samples were received October 26, 1994 and assayed in accordance with the protocol titled "Evaluation of WR 238605 as a Prophylactic Agent against Induced *P. falciparum* Malaria Infection in Healthy Non-immune Subjects: A Dose Ranging Study." Final analytical results were faxed to the COR on 11/21/94 and the report is in preparation.

WR5/P 95-3

Dog plasma samples were analyzed in accordance to the protocol titled "WR 238605 Multiple Drug Interaction Study in Beagle Dogs" as requested in a COR letter dated October 20, 1995. Results on 131 samples (and 18 dosing solutions not reported) received April 25 and November 25, 1996 for WR 238,605, mefloquine, chloroquine, monodesethyl-chloroquine, didesethyl-chloroquine, quinine, doxycycline, halofantrine and/or WR 178460 were faxed to the COR on March 20, 1997. On January 13-14, 1998 an additional 431 plasma samples and 21 vials of dosing solutions were received. On February 10, 1998 an additional 522 plasma samples and 6 vials of dosing solutions were received. Final results were e-mailed to the COR on December 30, 1998. A draft report was submitted for review on June 11, 1999.

WR5/P 96-2

Samples are to be analyzed as requested in a COR letter dated May 6, 1996 in accordance with the protocol titled "Dose-Ranging Study of the Safety and Efficacy of WR 238605 in the Prevention of Relapse of *Plasmodium vivax* Infection in Thailand." Final results on 266 human plasma and 260 human blood samples for WR 238,605 were faxed to the COR on March 6, 1997. On December 17, 1997, an additional 357 human plasma and 358 human blood samples were received (a recount shows that 1 sample less was received than originally believed). On March 2, 1998, final WR 238,605 results on 292 plasma and 292 blood (excluding samples from subjects dosed only with chloroquine) were faxed to the COR. Chloroquine blood and plasma analyses have been run and data is being evaluated. Additional samples are expected (COR fax dated March 5, 1998).

WR5/P 96-2 (Chlor)

Samples are to be analyzed for chloroquine and metabolites as requested in a COR letter dated May 6, 1996 in accordance with the protocol titled "Dose-Ranging Study of the Safety and Efficacy of WR 238605 in the Prevention of Relapse of *Plasmodium vivax* Infection in Thailand." See WR5/BP 96-2 above under WR 238605 for WR 238605 results. Chloroquine blood and plasma analyses for 357 human plasma and 358 human blood samples for chloroquine, monodesethylchloroquine, didesethylchloroquine were faxed to the COR on June 18, 2001. A draft report was submitted for review on August 2, 2001.

WR5/P 97-1

Samples were to be analyzed in accordance with the protocol titled " One Year Oral Toxicity Study of WR 238605 Succinate in Dogs." Samples (224 dog plasma) were received August 13, 1997. Final data was faxed to the COR September 26, 1997 and a draft report was submitted for review January 5, 1999 that was found acceptable as described in a COR Fax dated January 20, 1999. A signed report will be submitted with the final validation report.

WR 6026, WR 211,789 and WR 254,421

WR6/PU 94-3

Samples were received for routine analysis in accordance with the protocol titled "Clinical Trial of Oral WR6026•2HCl in Patients with Brazilian Visceral Leishmaniasis due to *L. chagasi*: Initial Dose Range Determination for Efficacy, Safety and Tolerance." Final data on 92 human plasma and 90 human urine samples was Faxed to the COR on January 27, 1997 and on 28 human plasma samples was Faxed to the COR on August 22, 1997. On November 13, 1997, 92 human sera samples were received and a request for analysis of previously received plasma/sera samples was made for WR 254421 determinations.

KEY RESEARCH ACCOMPLISHMENTS

Methods developed and validated for:

- Artelinic acid in human plasma (SR35, supplement 1)
- R & S Isomers of Halofantrine and WR 178460 in human plasma in (SR 28)

Sample results for:

- 357 results for chloroquine in human plasma,
  357 results for monodesethylchloroquine in human plasma,
  357 results for didesethylchloroquine in human plasma,
  358 results for chloroquine in human blood,
  358 results for monodesethylchloroquine in human blood,
  358 results for didesethylchloroquine in human blood,
  (in draft report WR5/BP 96-2 (Chlor)
- 12 results for artelinic acid in human plasma
  (in study Art/P 00-001)

REPORTABLE OUTCOMES

Quilei (Julie) Ren applied for and received employment and further training at Genesoft, Inc., 7300 Shoreline Ct., South San Francisco, CA, based in part on LC/MS/MS experience supported by this contract.

CONCLUSIONS

Using the procedures described in this report, we were able to work sequentially or simultaneously on development, validation and characterization of assays for WR 6026 (and its metabolites, WR 211789 and WR 254421), mefloquine (and its metabolite, WR 160972), *p*-aminoheptanophenone (and related compounds), WR 242511, halofantrine (and its metabolite, WR 178,460, and their stereoisomers), chloroquine (and its metabolites, monodesethylchloroquine and didesethylchloroquine), WR 243,251, WR 238,605, gentamicin, paromomycin and artelinic acid (and metabolites and artesunate). Work on routine analyses of biological specimens during this period was performed for studies that required determination of concentrations of artelinic acid, chloroquine (and its metabolites, monodesethylchloroquine and didesethylchloroquine), and stereoisomers of halofantrine (and its metabolite, WR 178,460). We worked on demonstrating sensitivity, specificity, linearity, lack of interferences, accuracy, and reproducibility of the analytical method, describing the extent of recovery for the method, and reporting on the stability of compounds of interest in specimens during storage and drug analysis to provide documentation in support of Investigational New Drug (IND) submissions to the Food and Drug Administration (FDA).

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APPENDIX A: VALIDATED METHODS

Study Report No. 28

Short Validation of a High Performance Liquid Chromatographic (HPLC) Method for the Determination of R & S Isomers of Halofantrine and WR 178460 in Human Plasma Samples

A. Materials

Test Compounds: R & S isomers of halofantrine and WR 178460

Drug Standards: Halofantrine, (WR 171669) bottle no. BM01792
WR 178460, bottle no. BM08577
(+)Halofantrine, (WR 216062) bottle no. BN46125
(-)Halofantrine, (WR 216063) bottle no. BN47051
(+)WR 280823, bottle no. BN78896
(-)WR 280691, bottle no. BN78903
were obtained from WRAIR

Internal Standard: Desmethylimipramine, obtained from Geigy Pharm.

Matrix: Human Plasma

Biological Matrix: Control and standard curve human plasma was obtained from UC Medical Center or Irwin Memorial Blood Bank, San Francisco. This matrix was used for daily preparation of standard and quality control samples. Prior to use, plasma was stored at 4°C. This material was found to be free of endogenous substances that would interfere with the quantitation of the drug or internal standard. Anticoagulant CPD was used.

Sample Storage: Temperature: Approximately -70°C

HPLC System

Detector: Shimadzu RF-535 Fluorescence HPLC monitor
Wavelengths: Excitation-300 nm; Emission-375 nm

Pump: Shimadzu LC-10AS Pump (Shimadzu Scientific Instruments, Inc., Columbus, MD) or equivalent

Injector: Waters Intelligent Sample Processor 710B (Waters Associates, Milford, MA) or equivalent

Data Acquisition: INTEGRATOR: Hewlett Packard Integrator 3390A, or equivalent.

Data Reduction: Internal standard method using peak height ratio (PHR). Weighted (1/y) linear regression of concentration (x-axis) vs. PHR (y-axis)

HPLC Conditions

Column:	Chiral AD, 5 µm, 25 cm X 4.6 mm, Daicel Chemical Industrial, Inc.
Column Temperature:	Room Temperature
WISP Temperature:	4°C
Mobile Phase:	Hexane, Ethanol, 2-butanol, diethylamine (100:1.5:1.3:0.1, v/v/v/v).
Flow Rate:	0.26 ml/min.
Detector Wavelength	Excitation-300 nm; Emission-375 nm
Detector Settings	Sensitivity-high; Range 32; Response-medium
Injection Volume:	10-35 µl
Laboratory Temperature:	60-80°F

Note: If necessary, HPLC conditions can be slightly modified to optimize the system.

Assay Parameters

Volume of Plasma Required for Assay:	0.50 ml
Assay Ranges:	halofantrine R & S isomers-10.0 to 400 ng/ml WR 178460 R & S isomers-15.0 to 600 ng/ml
Minimum Reportable Concentrations:	halofantrine R & S isomers-10.0 ng/ml WR 178460 R & S isomers-15.0 ng/ml

Chemicals and Supplies

Chemical/Solvents	**Grade**	**Supplier**
Hexane	HPLC	Fisher Scientific
Ethanol 200 proof dehydrated	alcohol reagent	Quantum Chemical
Water	Type I Reagent Grade	Nanopure, Barnstead
Methanol	HPLC	Fisher Scientific
2-Butanol		Sigma Chemical
Diethylamine		Sigma Chemical
Methyl *t*-butyl ether		Burdick and Jackson
Acetonitrile	HPLC	Fisher Scientific
Ammonium hydroxide	ACS plus	Fisher Scientific

B. Analytical Method

Human plasma samples (0.5 ml) were analyzed for R & S isomers of halofantrine and WR 178460 with an HPLC procedure that uses a fluorescence detector, a chiral column (4.6 x 250 mm, 5 µm particle size), a hexane/ethanol/2-butanol/diethyl-amine (100:1.5:1.3:0.1, v/v/v/v) mobile phase, and an HP

integrator. Sample preparation consisted of addition of 150 µl of desmethylimipramine internal standard (IS) methanol solution, precipitation with 1 ml of acetonitrile (followed by centrifugation for 1 minute and transfer and evaporation to 0.2 ml of the supernatant), followed by extraction with 5 ml methyl *t*-butyl ether, evaporation of the organic layer and reconstitution in mobile phase prior to injection onto the HPLC column. Standard curve and quality control (QC) samples were generated by spiking interference free human plasma samples with known amounts of R & S isomers of halofantrine and WR 178460 and IS. Standard curve, QC and assay samples were prepared as described, then 10-35 µl aliquots were injected into the HPLC system for chromatographic separation and subsequent fluorometric detection. The peak height ratios of R & S isomers of halofantrine and WR 178460 to IS were calculated for each sample from the measured peak heights obtained by HPLC. Finally, spiked concentrations and R or S isomer of halofantrine or WR 178460 to IS peak height ratios of the standard curve samples were fit by 1/y weighted least squares linear regression to the equation for the best straight line ($y = mx + b$, where y = peak height ratio and x = R or S isomers of halofantrine or WR 178460 concentrations), and drug concentrations in assay samples were calculated by these equation from the R or S isomer of halofantrine or WR 178460 to IS peak height ratios obtained by HPLC.

Calibration standards and validation samples with drug concentrations within the calibration range of the assay were analyzed to assess the performance of the assay. Calibration standards and validation samples were generated by spiking blank human plasma with racemic halofantrine and WR 178460.

Retention times (approximate, in decimal minutes) were 30.0: internal standard, 17.2: (+)halofantrine, 18.5: (-)halofantrine, 34.3: (-)WR 178460, and 38.8: (+)WR 178460.

Standard Curve and Control Solutions

STOCK SOLUTIONS: These solutions were stored in a 4°C refrigerator and protected against light.

Solution Type	Weight of Standard (mg)	Conversion Factor	QS Volume (ml)	Solvent	Conc. (mg/ml)
Halofantrine	1.074	0.932*	10	ethanol	0.100
WR 178460	1.080	0.924*	10	ethanol	0.10
Desmethylimipramine (IS)	1.6	1	10	ethanol	0.16

*= Molecular weights of halofantrine free base/halofantrine hydrochloride and WR 178460 free base/WR 178460 hydrochloride.

WORKING SOLUTIONS. These solutions were stored in a 4°C refrigerator and protected against light. Halofantrine and Halofantrine metabolite stock solutions were combined into a single solution (high working solution, HWS) and diluted with acetonitrile. The low working solution (LWS) was diluted from the HWS with acetonitrile.

Solution Type	Volume Diluted (ml)	Conc. Diluted (µg/ml)	QS Volume (ml)	Solvent	Conc. (µg/ml)
HWS					
[Halofantrine]	2.0	100	10	acetonitrile	20.0
[Halofantrine metabolite]	3.0	100			30
LWS					
[Halofantrine]	1.0	20.0	10	acetonitrile	2.00
[Halofantrine metabolite]		30			3.0
Desmethylimipramine (IS)	1:20	160	-	methanol	8.0

Calibration Standards and Quality Control Samples

The scheme for generating calibration standard and quality control (QC) samples for R & S isomers of halofantrine and WR 178460 is provided in the following tables.

Calibration Standards: Calibration standards were generated by spiking 0.5 ml blank human plasma specimens with halofantrine and WR 178460 standard curve solutions. This procedure is equivalent to addition of the masses of R & S isomers of halofantrine and WR 178460 shown below. Since 0.5 ml plasma samples are assayed and racemic mixtures of halofantrine and WR 178460 solutions were added, these amounts correspond to the nominal concentrations of each isomer shown below.

Generation of R & S Isomers of Halofantrine Standard Curve Calibrators

Sample	Standard Solution Volume (µl)	Spiking Solution Conc. (µg/ml)	Volume of Plasma (ml)	Mass of R or S Isomer (ng)	Standard Curve Sample Nominal R or S Isomer Conc. (ng/ml)
00*	0	0	0.500	0	0
0**	0	0	0.500	0	0
1	5.00	2.0	0.500	5	10
2	10.0	2.0	0.500	10	20
3	20.0	2.0	0.500	20	40
4	35.0	2.0	0.500	35	70
5	5.00	20.0	0.500	50	100
6	7.50	20.0	0.500	75	150
7	10.0	20.0	0.500	100	200
8	12.5	20.0	0.500	125	250
9	15.0	20.0	0.500	150	300
10	20.0	20.0	0.500	200	400

* 00 = Sample with no drug and no internal standard.
** 0 = Sample with no drug but with internal standard

Generation of R & S Isomers of WR 178460 Standard Curve Calibrators

Sample	Standard Solution Volume (µl)	Spiking Solution Conc. (µg/ml)	Volume of Plasma (ml)	Mass of R or S Isomer (ng)	Standard Curve Sample Nominal R or S Isomer Conc. (ng/ml)
00*	0	0	0.500	0	0
0**	0	0	0.500	0	0
1	5.00	3.0	0.500	7.5	15
2	10.0	3.0	0.500	15	30
3	20.0	3.0	0.500	30	60
4	35.0	3.0	0.500	52.5	105
5	5.00	30.0	0.500	75	150
6	7.50	30.0	0.500	112.5	225
7	10.0	30.0	0.500	150	300
8	12.5	30.0	0.500	187.5	375
9	15.0	30.0	0.500	225	450
10	20.0	30.0	0.500	300	600

Quality Control Samples: Quality control samples were generated by spiking 0.5 ml blank human plasma specimens as follows.

Generation of Precision Halofantrine Quality Control Samples

Sample ID	Control Solution Volume (µl)	Spiking Solution Conc. (µg/ml)	Mass of R or S Isomer Spiked (ng)	QC Sample Nominal R or S Isomer Concentration (ng/ml)
X-Low	10	2.00	10	20.0
Low	25	2.00	25	50.0
Med.	5	20.0	50	100
High	15	20.0	150	300

Generation of Precision WR 178460 Quality Control Samples

Sample ID	Control Solution Volume (µl)	Spiking Solution Conc. (µg/ml)	Mass of R or S Isomer Spiked (ng)	QC Sample Nominal R or S Isomer Concentration (ng/ml)
X-Low	10	3.0	15	30
Low	25	3.0	37.5	75
Med.	5	30	75	150
High	15	30	225	450

SAMPLE PREPARATION PROCEDURE

1. Pipette 0.50 ml plasma samples into 13 X 100 silanized tubes.

2. Spike standard curve and control samples with halofantrine and halofantrine metabolite as described above.

3. Vortex for 30s.

4. Add 150 µl of 8 µg/ml internal standard working solution. Add 150 µl of MeOH to 00 and vortex all samples for 30s.

5. Add 1 ml acetonitrile and then vortex for 1 min. Centrifuge 10 min at ~2000 *g*.

6. Transfer supernatant to 16 X 125 mm silanized tubes and evaporate to 0.2 ml volume.

7. Add 0.5 ml ammonium hydroxide and vortex for 30s.

8. Add 5 ml methyl *t*-butyl ether, vortex for 2 min, and centrifuge 10 min at ~2000 *g*.

9. Transfer organic layer to 13 X 100 silanized tubes and evaporate to dryness.

10. Reconstitute residues with 300 µl mobile phase (400 µl for samples with drug level more than 150 ng/ml). Vortex for 1 min.

11. Transfer to silanized glass inserts and inject 10-35 µl onto column.

Study Report No. 35, Supplement I

Validation of a Liquid Chromatographic/Mass Spectrometric/Mass Spectrometric (LC/MS/MS) Method for the Determination of Artelinic Acid in Human Plasma Samples

A. Materials

Test Compound:	Artelinic Acid
Drug Standards:	Artelinic Acid (WR255663AK), bottle no. BM04131, was obtained from WRAIR.
Internal Standard:	Indomethacin, Lot number 19C-0312, was purchased from Sigma Chemical Co.
Matrix:	Human plasma
Biological Matrix:	Control and standard curve human plasma was obtained the UCSF Medical Center and the Blood Center of the Pacific, San Francisco, CA. This matrix was used for daily preparation of standard and quality control samples. Prior to use, plasma was stored at -20°C. This material was found to be free of endogenous substances that would interfere with the quantitation of the drug or internal standard. CPD or CPDA-1 were used as an anticoagulant.
Sample Storage:	Temperature: Approximately -20°C

LC/MS/MS System

Detector:	API III PE-Sciex (Perkin-Elmer, Norwalk, CT)
Pump:	Shimadzu LC-10AD Pump (Shimadzu Scientific Instruments, Inc., Columbus, MD) or equivalent
Injector:	Waters Intelligent Sample Processor 717 Plus (Waters Associates, Milford, MA) or equivalent
Data Acquisition:	Macintosh Quadra 800 (Apple, Cupertino, CA) Mac Spec 3.3 Software (Perkin-Elmer, Norwalk, CT) RAD 2.4 Software (Perkin-Elmer, Norwalk, CT)
Data Reduction:	Internal standard method using peak area ratio (PAR). Weighted (1/y) linear regression of concentration (x-axis) vs. PAR (y-axis)

LC/MS/MS Conditions

Column:	Hypersil C8, 3 µm particle size, 4.6 X 50 mm (Keystone Scientific, Inc., Bellefonte, PA) or equivalent
Column Temperature:	Room Temperature
Mobile Phase:	35% CH_3CN, 35% MeOH, 0.1% trifluoroacetic acid, (TFA). The mobile phase was prepared by mixing 1400 ml CH_3CN, 1400 ml MeOH, 1200 ml of water, and adding 4 ml of TFA to yield about 4 liters. The resulting solution was filtered through a 5 micron filter and degassed under vacuum prior to use.
Flow Rate:	1.0 ml/min.
Sample Inlet Mode:	Heated Nebulizer
Ionization Mode:	APCI/Positive Ionization
Discharge Current:	+ 3 µA
Curtain Gas Flow Rate:	1.2 L/min (N_2 = 99.999%)
Nebulizer Pressure:	80 psi (Ultra Zero Air)
Auxiliary Flow Rate:	2.0 L/min (Ultra Zero Air)
CAD Gas:	250 x 10^{12} molecules/cm^2 (9.99% N_2/Ar)
Interface Heater Temperature:	55°C
Heated Nebulizer Temperature Controller:	450°C
Laboratory Temperature:	60-80°F

Mass Scanning Mode: MRM (Multiple Reaction Monitoring)

Artelinic Acid (High Range):	373 - 220 *m/z*
Artelinic Acid (Low Range):	373 - 162 *m/z*
Indomethacin (I.S.):	359 - 139 *m/z*

Assay Parameters

Volume of Plasma Required for Assay:	100 µl
Assay Ranges:	Artelinic Acid Low Range: 4.00 to 240 ng/ml Artelinic Acid High Range: 60.0 to 4800 ng/ml
Minimum Reportable Concentration:	4.00 ng/ml for Artelinic Acid

Chemicals and Supplies

Chemical/Solvents	Grade	Supplier
Acetonitrile	HPLC	Fisher Scientific
Trifluoroacetic Acid	Reagent	Sigma Chemical
Water	Type I Reagent Grade	Nanopure, Barnstead
Methanol	Optima	Fisher Scientific

B. Analytical Method

Human plasma samples (100 µl) were analyzed for Artelinic Acid with an LC/MS/MS procedure in a PE Sciex-API III system equipped with a C8 column (4.6 x 50 mm, 3 µm particle size), 35% CH_3CN, 35% MeOH, 0.1% TFA mobile phase and mass spectrometric detection with sample inlet by heated nebulizer, positive ionization by APCI (atmospheric pressure chemical ionization) and mass scanning by MRM (Multiple Reaction Monitoring) analysis. Sample preparation consisted of addition of 200 µl of acetonitrile containing indomethacin internal standard (IS), mixing of the mixture for 1 minute, centrifugation for 5 minutes and transfer of the supernatant to an autosampler injection vial prior to separation by LC/MS/MS. Standard curve and quality control (QC) samples were generated by spiking interference free human plasma samples with known amounts of Artelinic Acid and IS. Standard curve, QC and assay samples were prepared as described, then ~40 µl aliquots were injected into the LC/MS/MS system for chromatographic separation and subsequent mass spectrometric detection. The peak area ratios of Artelinic Acid (high range daughter ion at 220 *m/z* and low range daughter at 162 *m/z* from parent ion at 373 *m/z*) to IS (daughter ion at 139 *m/z* from parent ion at 359 *m/z*) were calculated for each sample from the measured peak areas obtained by LC/MS/MS. Finally, spiked concentrations and (Artelinic Acid to IS) peak area ratios of the standard curve samples were fit by 1/y weighted least squares linear regression to the equations for the best straight lines ($y = mx + b$, where y = peak area ratio and x = Artelinic Acid concentrations), and drug concentrations in assay samples were calculated by these equations from the Artelinic Acid to IS peak area ratios obtained by LC/MS/MS.

Calibration standards and validation samples with drug concentrations within the calibration range of the assay were analyzed to assess the performance of the assay. Calibration standards and validation samples were generated by spiking blank human plasma with Artelinic Acid.

Retention times (approximate, in decimal minutes) were internal standard 1.20 and Artelinic Acid 1.90. The time between injections was 4-5 minutes.

Standard and Control Solutions

STOCK SOLUTIONS: These solutions were stored in a 4°C refrigerator.

Solution Type	Weight of Standard (mg)	Adjustment Factor*	Solvent Volume (ml)	Solvent	Conc. (mg/ml)
Artelinic Acid Standard Curve	5.34	0.95	5.073	MeOH	1.00
Artelinic Acid Control	5.47	0.95	5.197	MeOH	1.00
Indomethacin (IS)	5.28	1	5.28	MeOH	1.00

* = Purity.

WORKING SOLUTIONS. These solutions were stored in a 4°C refrigerator.

Solution Type	Volume Diluted (ml)	Conc. Diluted (µg/ml)	QS Volume (ml)	Solvent	Conc. (µg/ml)
Standard Curve High	0.200	1000	10.0	50% CH_3CN	20.0
Standard Curve Medium	1.00	20.0	10.0	50% CH_3CN	2.00
Standard Curve Low	1.00	2.00	10.0	50% CH_3CN	0.200
Control High	0.200	1000	10.0	50% CH_3CN	20.0
Control Medium	1.00	20.0	10.0	50% CH_3CN	2.00
Control Low	1.00	2.00	10.0	50% CH_3CN	0.200
Indomethacin Substock IS	0.500	1000	50.0	100% CH_3CN	10.0
Indomethacin Working IS	0.100	10.0	100	100% CH_3CN	0.0100

Calibration Standards and Quality Control Samples

The scheme for generating calibration standard and quality control (QC) samples for Artelinic Acid is provided in the following tables.

Calibration Standards: Calibration standards were generated by spiking 100 µl blank human plasma specimens with artelinic acid standard curve solutions. This procedure is equivalent to addition of the masses of artelinic acid shown below. Since 100 µl plasma samples are assayed, these amounts correspond to the nominal concentrations shown below. Vortex for 10 seconds.

Generation of Artelinic Acid Standard Curve Calibrators

Sample	Curve Range	Volume Spiked (µl)	Spiking Solution Concentration (µg/ml)	Mass Spiked (ng)	Standard Curve Sample Nominal Concentration (ng/ml)
00*		0	0	0	0
0**		0	0	0	0
1	Low	2	0.200	0.400	4
2	Low	4	0.200	0.800	8
3	Low	8	0.200	1.60	16
4	Low	16	0.200	3.20	32
5	Low-High	3	2.00	6.00	60
6	Low-High	6	2.00	12.0	120
7	Low-High	12	2.00	24.0	240
8	High	24	2.00	48.0	480
9	High	6	20.0	120	1200
10	High	12	20.0	240	2400
11	High	24	20.0	480	4800

Quality Control Samples: Quality control samples were generated by spiking 100 µl blank human plasma specimens as follows.

Generation of Artelinic Acid Precision Quality Control Samples

	Volume Spiked (µl)	Spiking Solution Conc. (µg/ml)	Plasma Volume (µl)	Artelinic Acid Quality Control Sample Nominal Conc. (ng/ml)
Low	4.00	0.200	100	8.00
Medium	15.0	0.200	100	30.0
High	6.00	2.00	100	120
xHigh	4.00	20.0	100	800
xxHigh	20.0	20.0	100	4000

SAMPLE PREPARATION PROCEDURE

1. Pipet 100 µl of blank human plasma into a 13 x 100 tube.
2. Spike standard curve samples as described above and vortex for 30 seconds.
3. Add 200 µl of internal standard working solution and vortex for 1 minute.
4. Centrifuge for 5 minutes at full speed.
5. Transfer to autosampler vial and inject ~40 µl onto the column.

* 00 = Sample with no drug and no internal standard.
** 0 = Sample with no drug but with internal standard.

APPENDIX B: ROUTINE ASSAY RESULTS

WR5/BP 96-2 (Chlor)

Analytical Data, WR5/BP 96-2 (Chlor) Chloroquine, Didesethylchloroquine and Monodesethylchloroquine Concentrations in Human Plasma and Blood

Sample ID	Plasma CHLORO-QUINE (ng/ml)	Plasma DI-CHLORO (ng/ml)	Plasma MONO-CHLORO (ng/ml)	Blood CHLORO-QUINE (ng/ml)	Blood DI-CHLORO (ng/ml)	Blood MONO-CHLORO (ng/ml)
21-D(6/08/13)	137	*	42.2	444	27.7	168
21-D(6/08/14)	114	*	48.9	482	26.8	157
21-D(6/08/15)	69.8	*	30.7	405	25.8	135
21-D(6/08/21)	56.5	*	65.7	97.8	*	57.6
21-D(6/08/28)	*	*	26.9	44.4	*	33.9
21-D(6/09/11)	*	*	*	*	*	*
21-D(6/09/25)	*	*	*	*	*	*
21-D(6/10/09)	*	*	*	*	*	*
21-D(6/11/06)	*	*	*	*	*	*
22-B(6/08/15)	123	*	39.5	484	45.5	174
22-B(6/08/16)	136	30.5	117	414	47.2	162
22-B(6/08/17)	97.5	26.2	91.7	306	40.7	133
22-B(6/08/23)	55.9	39.1	115	109	34.1	87.5
22-B(6/08/26)	26.6	22.7	70.1	90.3	33.3	77.9
22-B(6/09/13)	*	*	*	*	*	23.8
22-B(6/09/27)	*	*	*	*	*	*
23-C(6/08/16)	NS	NS	NS	822	167	499
23-C(6/08/17)	187	72.2	203	711	183	487
23-C(6/08/18)	160	84.0	211	582	170	427
24-A(6/08/20)	289	89.4	254	797	113	322
24-A(6/08/21)	202	66.1	183	849	131	364
24-A(6/08/22)	172	91.7	217	622	117	299
24-A(6/08/28)	57.5	44.6	91.6	227	55.0	121
24-A(6/09/04)	*	*	40.1	91.0	27.4	51.6
24-A(6/09/18)	*	*	*	21.8	*	24.3
24-A(6/10/02)	*	*	*	*	*	*
24-A(6/10/16)	*	*	*	*	*	*
24-A(6/11/13)	*	*	*	*	*	*
24-A(6/12/11)	*	*	*	*	*	*
24-A(7/01/08)	*	*	*	*	*	*
24-A(7/02/05)	*	*	*	*	*	*
25-B(6/08/25AM)	332	116	355	719	117	368
25-B(6/08/25PM)	355	106	371	897	124	465
25-B(6/08/26)	262	85.6	281	645	100	333
25-B(6/08/30)	91.3	40.9	116	269	55.7	147
25-B(6/09/08)	26.7	*	33.3	102	26.4	52.0
25-B(6/09/22)	*	*	*	34.8	*	27.8
25-B(6/10/06)	*	*	*	20.1	*	22.4
25-B(6/10/20)	*	*	*	*	*	*

Analytical Data, WR5/BP 96-2 (Chlor) Chloroquine, Didesethylchloroquine and Monodesethylchloroquine Concentrations in Human Plasma and Blood (Continued)

Sample ID	Plasma CHLORO-QUINE (ng/ml)	Plasma DI-CHLORO (ng/ml)	Plasma MONO-CHLORO (ng/ml)	Blood CHLORO-QUINE (ng/ml)	Blood DI-CHLORO (ng/ml)	Blood MONO-CHLORO (ng/ml)
25-B(6/11/09)	*	*	*	*	*	*
25-B(6/12/15)	*	*	*	*	*	*
25-B(7/01/12)	*	*	*	*	*	*
25-B(7/02/09)	*	*	*	*	*	*
27-C(6/08/28)	289	34.8	193	732	69.5	360
27-C(6/08/29)	254	50.7	240	624	84.9	385
27-C(6/08/30)	181	39.1	179	606	84.4	340
27-C(6/09/03)	79.7	31.2	110	248	54.7	167
27-C(6/09/08)	45.8	24.6	85.7	132	38.1	113
28-A(6/08/30)	179	74.2	186	430	91.1	208
30-A(6/08/30)	173	65.4	176	410	79.4	203
30-A(6/08/31)	131	62.4	164	371	85.4	205
30-A(6/09/01)	96.9	53.5	126	394	94.3	204
31-D(6/09/02)	356	49.3	275	689	74.5	356
31-D(6/09/03)	317	64.2	311	534	73.2	312
31-D(6/09/04)	169	40.6	178	473	67.3	258
31-D(6/09/08)	55.6	*	63.4	234	47.9	152
31-D(6/09/13)	44.2	26.7	86.5	95.5	35.6	88.3
31-D(6/09/24)	*	*	47.5	33.1	*	42.8
32-C(6/09/03)	194	37.1	150	464	43.5	166
32-C(6/09/04)	187	47.8	154	556	50.8	178
32-C(6/09/05)	128	42.4	125	467	46.2	157
32-C(6/09/14)	51.9	41.9	105	93.4	34.8	84.3
32-C(6/09/18)	30.8	33.9	71.2	51.8	27.9	58.7
32-C(6/10/02)	*	*	29.2	*	*	21.8
32-C(6/10/16)	*	*	*	*	*	*
32-C(6/10/30)	*	*	*	*	*	*
32-C(6/11/27)	*	*	*	*	*	*
32-C(6/12/25)	*	*	*	*	*	*
33-D(6/09/06)	260	44.1	353	730	51.9	436
33-D(6/09/07)	97.3	*	114	541	48.4	381
33-D(6/09/08)	177	46.1	356	430	55.6	352
33-D(6/09/17)	63.3	26.0	183	97.0	24.6	124
33-D(6/09/21)	*	*	36.4	60.8	*	78.9
33-D(6/10/05)	*	*	40.3	*	*	26.1
33-D(6/10/19)	*	*	21.3	*	*	20.1
33-D(6/10/30)	*	*	*	*	*	*
34-B(6/09/09)	364	113	366	625	128	366
34-B(6/09/10)	218	85.1	238	693	152	418
34-B(6/09/11)	275	168	425	611	152	395
34-B(6/09/13)	109	83.9	198	374	113	248
34-B(6/09/20)	41.3	46.2	104	138	50.2	97.3
34-B(6/10/08)	*	*	23.1	22.2	22.3	31.0

Analytical Data, WR5/BP 96-2 (Chlor) Chloroquine, Didesethylchloroquine and Monodesethylchloroquine Concentrations in Human Plasma and Blood (Continued)

Sample ID	Plasma CHLORO-QUINE (ng/ml)	Plasma DI-CHLORO (ng/ml)	Plasma MONO-CHLORO (ng/ml)	Blood CHLORO-QUINE (ng/ml)	Blood DI-CHLORO (ng/ml)	Blood MONO-CHLORO (ng/ml)
34-B(6/10/22)	*	*	*	*	*	21.5
35-C(6/09/10)	245	27.9	112	405	47.1	147
35-C(6/09/12)	167	34.2	112	364	47.5	146
35-C(6/09/14)	90.4	*	55.7	286	43.2	121
35-C(6/09/21)	48.6	*	53.8	124	30.0	68.2
35-C(6/09/25)	38.9	*	48.5	80.2	20.8	51.1
35-C(6/10/09)	*	*	22.6	33.4	*	23.5
35-C(6/10/24)	*	*	*	*	*	*
35-C(6/11/06)	*	*	*	*	*	*
35-C(6/12/04)	*	*	*	*	*	*
35-C(7/01/02)	*	*	*	*	*	*
35-C(7/01/29)	*	*	*	*	*	*
35-C(7/02/26)	*	*	*	*	*	*
36-(6/09/16)	279	48.3	169	687	70.1	256
37-B(6/09/19)	171	*	82.7	1050	61.7	430
37-B(6/09/21)	117	*	87.4	887	76.8	430
37-B(6/09/23)	123	31.3	144	700	74.3	357
37-B(6/09/28)	72.3	*	81.0	267	35.6	142
37-B(6/09/30)	38.2	*	40.6	229	28.1	114
38-D(6/09/20)	132	22.1	80.8	656	70.0	265
38-D(6/09/21)	77.4	*	50.8	534	62.9	203
38-D(6/09/22)	88.2	31.3	88.2	443	61.4	195
38-D(6/09/28)	26.7	*	36.7	86.9	34.9	67.5
38-D(6/10/05)	*	*	36.4	35.5	*	30.5
38-D(6/10/19)	*	*	*	*	*	*
38-D(6/11/03)	*	*	*	*	*	*
39-A(6/09/21)	166	*	83.4	575	38.0	167
39-A(6/09/23)	122	21.6	89.0	306	33.3	121
39-A(6/09/25)	73.0	*	63.3	207	30.9	87.3
39-A(6/10/02)	20.9	*	35.9	54.8	*	39.3
39-A(6/10/06)	*	*	29.1	39.5	*	27.2
39-A(6/10/20)	*	*	*	*	*	*
39-A(6/11/03)	*	*	*	*	*	*
39-A(6/11/22)	*	*	*	*	*	*
39-A(6/12/15)	*	*	*	*	*	*
39-A(7/01/12)	*	*	*	*	*	*
39-A(7/02/09)	*	*	*	*	*	*
39-A(7/03/09)	*	*	*	*	*	*
40-A(6/09/21)	186	*	89.4	741	86.5	506
40-A(6/09/23)	326	74.6	396	500	81.2	394
41-B(6/09/21)	226	39.1	149	644	83.8	313
41-B(6/09/23)	208	59.5	182	618	85.6	294
41-B(6/09/25)	154	58.5	161	484	77.7	223
41-B(6/09/30)	47.9	28.2	78.1	152	39.5	86.3

Analytical Data, WR5/BP 96-2 (Chlor) Chloroquine, Didesethylchloroquine and Monodesethylchloroquine Concentrations in Human Plasma and Blood (Continued)

Sample ID	Plasma CHLORO-QUINE (ng/ml)	Plasma DI-CHLORO (ng/ml)	Plasma MONO-CHLORO (ng/ml)	Blood CHLORO-QUINE (ng/ml)	Blood DI-CHLORO (ng/ml)	Blood MONO-CHLORO (ng/ml)
41-B(6/10/06)	21.7	20.3	45.2	87.8	29.7	55.5
41-B(6/10/20)	*	*	*	29.9	*	31.8
43-C(6/09/23)	224	82.7	198	573	110	267
43-C(6/09/25)	127	84.8	168	469	122	242
43-C(6/09/27)	88.3	85.8	143	525	132	228
43-C(6/09/29)	51.1	47.3	86.7	221	94.3	147
43-C(6/10/04)	40.4	41.4	70.3	94.0	57.5	83.0
43-C(6/10/22)	*	*	*	*	21.5	20.3
43-C(6/11/05)	*	*	*	*	*	*
43-C(6/11/19)	*	*	*	*	*	*
43-C(6/12/17)	*	*	*	*	*	*
43-C(7/01/14)	*	*	*	*	*	*
43-C(7/02/11)	*	*	*	*	*	*
43-C(7/03/11)	*	*	*	*	*	*
44-D(6/09/25)	286	36.5	186	577	81.6	334
44-D(6/09/26)	208	41.2	174	455	81.1	315
44-D(6/09/27)	107	22.3	101	157	47.4	154
44-D(6/10/01)	72.6	30.1	102	146	45.3	125
44-D(6/10/03)	47.2	22.7	75.1	100	33.2	81.6
44-D(6/10/24)	*	*	*	*	*	*
45-A(6/09/26)	218	42.8	190	516	57.8	235
45-A(6/09/28)	102	*	84.6	571	67.7	243
45-A(6/09/30)	177	67.3	234	410	59.7	214
46-C(6/09/30)	404	58.3	340	712	61.5	337
46-C(6/10/02)	353	129	535	598	99.8	377
46-C(6/10/04)	235	123	405	499	96.8	320
46-C(6/10/08)	167	130	415	272	82.2	252
46-C(6/10/11)	107	93.6	325	173	71.3	213
46-C(6/10/29)	*	*	56.5	28.8	*	42.2
46-C(6/11/12)	*	*	40.4	*	*	30.3
46-C(6/11/26)	*	*	37.0	*	*	*
46-C(6/12/24)	*	*	*	*	*	*
46-C(7/01/21)	*	*	*	*	*	*
47-B(6/10/03)	401	74.5	393	509	48.5	248
47-B(6/10/05)	240	60.3	285	424	47.2	208
47-B(6/10/07)	190	53.2	242	298	41.7	158
47-B(6/10/11)	81.2	31.6	140	138	26.5	86.1
47-B(6/10/18)	38.8	*	75.4	40.7	*	43.9
47-B(6/11/01)	*	*	36.9	*	*	*
47-B(6/11/15)	*	*	*	*	*	*
47-B(6/11/29)	*	*	*	*	*	*
47-B(6/12/27)	*	*	*	*	*	*
47-B(7/01/24)	*	*	*	*	*	*
47-B(7/02/21)	*	*	*	*	*	*

Analytical Data, WR5/BP 96-2 (Chlor) Chloroquine, Didesethylchloroquine and Monodesethylchloroquine Concentrations in Human Plasma and Blood (Continued)

Sample ID	Plasma CHLORO-QUINE (ng/ml)	Plasma DI-CHLORO (ng/ml)	Plasma MONO-CHLORO (ng/ml)	Blood CHLORO-QUINE (ng/ml)	Blood DI-CHLORO (ng/ml)	Blood MONO-CHLORO (ng/ml)
47-B(7/03/21)	*	*	*	*	*	*
48-D(6/10/03)	599	113	494	968	128	552
48-D(6/10/04)	390	96.0	367	605	115	394
48-D(6/10/05)	377	140	482	1180	146	548
48-D(6/10/11)	81.6	59.7	149	174	68.9	157
48-D(6/10/18)	36.3	35.8	111	47.1	36.5	72.8
48-D(6/11/01)	*	*	38.8	*	*	25.0
48-D(6/11/16)	*	*	*	*	*	*
49-C(6/10/04)	330	27.3	327	466	37.7	297
49-C(6/10/05)	262	28.7	258	545	46.8	331
49-C(6/10/06)	348	50.1	423	523	51.2	325
49-C(6/10/12)	154	38.1	251	274	48.7	255
49-C(6/10/19)	50.3	*	80.9	114	32.6	132
49-C(6/11/02)	*	*	*	39.7	*	47.9
49-C(6/11/16)	*	*	24.2	26.3	*	42.9
49-C(6/11/30)	*	*	20.0	*	*	31.2
49-C(6/12/27)	*	*	27.4	*	*	20.4
49-C(7/01/25)	*	*	*	*	*	*
49-C(7/02/22)	*	*	*	*	*	*
49-C(7/03/22)	*	*	*	*	*	*
51-C(6/10/10)	227	198	266	350	153	185
51-C(6/10/11)	135	152	163	313	175	177
51-C(6/10/12)	71.5	80.1	77.5	210	142	135
51-C(6/10/16)	90.1	208	210	111	107	97.3
51-C(6/10/21)	58.7	189	203	58.7	94.3	87.0
51-C(6/11/08)	*	39.6	37.8	*	36.9	*
51-C(6/11/22)	*	64.3	50.9	*	22.9	*
51-C(6/12/06)	*	*	*	*	*	*
51-C(7/01/03)	*	*	*	*	*	*
51-C(7/01/31)	*	*	*	*	*	*
51-C(7/02/28)	*	*	*	*	*	*
51-C(7/03/28)	*	*	*	*	*	*
52-A(6/10/10)	207	56.9	165	447	83.7	240
52-A(6/10/11)	125	33.3	92.3	436	96.2	237
52-A(6/10/12)	112	44.7	117	557	99.8	265
52-A(6/10/16)	92.9	83.9	158	191	67.2	111
52-A(6/10/21)	26.9	22.2	43.5	52.9	27.7	31.3
52-A(6/11/08)	*	*	*	*	*	*
52-A(6/11/24)	*	*	*	*	*	*
52-A(6/12/06)	*	*	*	*	*	*
52-A(7/01/03)	*	*	*	*	*	*
52-A(7/02/01)	*	*	*	*	*	*
52-A(7/03/01)	*	*	*	*	*	*
52-A(7/03/29)	*	*	*	*	*	*

Analytical Data, WR5/BP 96-2 (Chlor) Chloroquine, Didesethylchloroquine and Monodesethylchloroquine Concentrations in Human Plasma and Blood (Continued)

Sample ID	Plasma CHLORO-QUINE (ng/ml)	Plasma DI-CHLORO (ng/ml)	Plasma MONO-CHLORO (ng/ml)	Blood CHLORO-QUINE (ng/ml)	Blood DI-CHLORO (ng/ml)	Blood MONO-CHLORO (ng/ml)
53-A(6/10/12)	207	29.6	182	609	42.1	252
53-A(6/10/13)	82.8	*	58.3	573	41.3	242
53-A(6/10/14)	69.9	*	62.8	568	44.6	232
53-A(6/10/23)	*	*	29.9	60.7	*	28.7
53-A(6/10/27)	*	*	29.7	38.1	*	*
54-B(6/10/12)	194	*	143	465	30.8	200
54-B(6/10/14)	154	25.3	168	321	30.1	170
54-B(6/10/16)	144	34.4	200	309	29.1	146
54-B(6/10/21)	34.3	*	57.0	121	*	53.1
54-B(6/10/23)	37.9	*	65.5	89.3	*	43.7
54-B(6/11/10)	*	*	*	*	*	*
54-B(6/11/24)	*	*	*	*	*	*
54-B(6/12/08)	*	*	*	*	*	*
54-B(7/01/05)	*	*	*	*	*	*
54-B(7/03/04)	*	*	*	*	*	*
54-B(7/03/30)	*	*	*	*	*	*
55-A(6/10/12)	164	70.5	158	573	204	423
55-A(6/10/14)	169	118	218	501	182	334
55-A(6/10/16)	85.4	72.1	122	274	131	203
55-A(6/10/18)	93.8	90.8	163	158	87.9	137
55-A(6/10/23)	*	*	23.2	75.8	34.8	46.1
55-A(6/11/10)	*	*	*	*	*	*
55-A(6/11/26)	*	*	*	*	*	*
55-A(6/12/08)	*	*	*	*	*	*
55-A(7/01/05)	*	*	*	*	*	*
55-A(7/02/02)	*	*	*	*	*	*
56-A(6/10/13)	134	*	45.3	460	33.7	161
56-A(6/10/15)	202	60.8	238	480	47.5	206
56-A(6/10/17)	169	74.8	259	323	49.3	160
56-A(6/10/21)	90.2	58.7	197	205	40.9	112
56-A(6/10/24)	33.4	24.6	73.4	118	26.1	65.9
57-A(6/10/14)	258	57.4	188	478	75.2	222
57-A(6/10/15)	217	79.4	221	420	77.9	213
57-A(6/10/16)	174	86.6	196	302	74.2	157
57-A(6/10/22)	84.9	59.4	119	153	48.6	77.8
57-A(6/10/29)	25.0	*	30.5	62.3	22.8	27.0
57-A(6/11/12)	*	*	27.1	27.0	*	*
59-B(6/10/18)	228	35.5	249	454	30.8	210
59-B(6/10/19)	148	27.3	178	440	36.6	214
59-B(6/10/20)	115	29.0	160	348	36.2	178
59-B(6/10/26)	64.1	24.9	133	102	21.7	74.6
59-B(6/10/29)	35.1	*	74.8	75.4	*	60.9
59-B(6/11/16)	*	*	20.5	*	*	*
59-B(6/11/30)	*	*	*	*	*	*

Analytical Data, WR5/BP 96-2 (Chlor) Chloroquine, Didesethylchloroquine and Monodesethylchloroquine Concentrations in Human Plasma and Blood (Continued)

Sample ID	Plasma CHLORO-QUINE (ng/ml)	Plasma DI-CHLORO (ng/ml)	Plasma MONO-CHLORO (ng/ml)	Blood CHLORO-QUINE (ng/ml)	Blood DI-CHLORO (ng/ml)	Blood MONO-CHLORO (ng/ml)
59-B(6/12/14)	*	*	*	*	*	*
59-B(7/01/12)	*	*	*	*	*	*
59-B(7/02/07)	*	*	*	*	*	*
59-B(7/03/07)	*	*	*	*	*	*
59-B(7/04/04)	*	*	*	*	*	*
60-C(6/10/18)	280	90.5	320	524	76.4	283
60-C(6/10/19)	250	103	338	638	103	341
60-C(6/10/20)	208	108	344	536	110	350
60-C(6/10/26)	59.4	48.5	120	166	51.2	112
60-C(6/11/02)	26.4	20.5	45.6	58.5	25.8	46.7
60-C(6/11/16)	*	*	24.8	21.9	*	*
60-C(6/11/30)	*	*	36.0	*	*	*
60-C(6/12/14)	*	*	25.4	*	*	*
60-C(7/01/12)	*	*	*	*	*	*
60-C(7/02/09)	*	*	*	*	*	*
60-C(7/03/09)	*	*	*	*	*	*
60-C(7/04/06)	*	*	*	*	*	*
61-D(6/10/21)	370	34.7	290	608	40.4	323
61-D(6/10/22)	236	33.4	242	537	42.8	305
61-D(6/10/23)	147	23.5	136	595	45.8	321
61-D(6/10/29)	73.5	*	94.2	182	22.0	101
61-D(6/11/05)	30.6	*	46.4	78.1	*	35.0
61-D(6/11/19)	*	*	32.2	25.9	*	*
64-A(6/11/08)	385	125	288	697	127	310
64-A(6/11/10)	124	53.6	120	445	108	228
64-A(6/11/12)	151	117	220	594	145	288
64-A(6/11/16)	39.3	34.8	59.0	192	69.2	131
64-A(6/11/19)	36.2	45.1	81.8	80.2	42.4	63.3
64-A(6/12/07)	*	27.2	52.4	*	*	*
64-A(6/12/22)	*	*	*	*	*	*
64-A(7/01/05)	*	*	*	*	*	*
64-A(7/02/02)	*	*	*	*	*	*
64-A(7/03/02)	*	*	*	*	*	*
64-A(7/03/30)	*	*	*	*	*	*
64-A(7/04/27)	*	*	*	*	*	*
65-A(6/11/09)	259	43.1	156	808	76.9	295
65-A(6/11/10)	173	48.9	152	676	81.0	291
65-A(6/11/11)	194	74.9	226	594	90.6	280
65-A(6/11/20)	28.3	*	41.6	98.8	28.9	47.6
65-A(6/11/24)	*	*	31.3	71.5	21.9	29.1
65-A(6/12/08)	*	*	*	*	*	*
65-A(6/12/22)	*	*	*	*	*	*
65-A(7/01/05)	*	*	*	*	*	*
65-A(7/02/02)	*	*	*	*	*	*

Analytical Data, WR5/BP 96-2 (Chlor) Chloroquine, Didesethylchloroquine and Monodesethylchloroquine Concentrations in Human Plasma and Blood (Continued)

Sample ID	Plasma CHLORO-QUINE (ng/ml)	Plasma DI-CHLORO (ng/ml)	Plasma MONO-CHLORO (ng/ml)	Blood CHLORO-QUINE (ng/ml)	Blood DI-CHLORO (ng/ml)	Blood MONO-CHLORO (ng/ml)
65-A(7/03/02)	*	*	*	*	*	*
65-A(7/04/30)	*	*	*	*	*	*
66-B(6/11/15)	252	53.6	133	586	76.1	192
66-B(6/11/16)	162	43.6	116	580	86.6	214
66-B(6/11/17)	127	37.1	96.5	554	85.9	217
66-B(6/11/19)	114	54.3	107	382	62.4	132
66-B(6/11/26)	25.2	*	35.7	137	27.1	43.4
66-B(6/12/14)	*	*	*	*	*	*
66-B(6/12/28)	*	*	*	*	*	*
66-B(7/01/11)	*	*	*	*	*	*
67-B(6/11/18)	227	31.3	128	407	38.2	145
67-B(6/11/20)	117	22.9	85.5	204	31.8	83.0
67-B(6/11/22)	86.4	23.4	71.6	199	30.9	75.6
67-B(6/11/27)	68.4	20.6	76.6	142	*	47.6
67-B(6/12/03)	20.9	*	28.2	60.3	*	*
69-A(6/11/25)	210	108	152	448	144	209
69-A(6/11/27)	106	101	127	393	134	170
69-A(6/11/29)	46.5	44.7	47.0	250	103	123
69-A(6/12/06)	*	33.5	37.6	53.3	36.9	37.1
69-A(6/12/10)	*	*	*	27.1	28.0	25.8
69-A(6/12/24)	*	*	*	*	*	*
69-A(7/01/07)	*	*	*	*	*	*
69-A(7/01/21)	*	*	*	*	*	*
69-A(7/02/19)	*	*	*	*	*	*
69-A(7/03/18)	*	*	*	*	*	*
70-D(6/12/03)	198	160	178	608	234	276
70-D(6/12/04)	144	157	164	453	235	239
70-D(6/12/05)	129	194	186	378	209	199
70-D(6/12/09)	59.9	126	124	133	141	119
70-D(6/12/11)	31.0	77.1	70.8	78.4	98.8	77.8
71-B(6/12/10)	210	33.7	227	295	38.7	214
71-B(6/12/12)	176	49.7	253	223	43.6	187
71-B(6/12/14)	102	31.1	164	153	41.0	160
71-B(6/12/18)	59.6	21.9	106	77.3	25.4	79.3
71-B(6/12/25)	*	*	*	41.2	*	37.0
71-B(7/01/08)	*	*	20.0	*	*	*
71-B(7/01/22)	*	*	*	*	*	*
71-B(7/02/05)	*	*	*	*	*	*
71-B(7/03/05)	*	*	*	*	*	*
71-B(7/04/02)	*	*	*	*	*	*
73-D(6/12/21)	88.0	35.1	99.7	575	154	430
73-D(6/12/22)	146	98.1	241	513	153	406
73-D(6/12/23)	166	109	292	399	119	326
73-D(6/12/29)	97.7	75.9	198	188	74.9	182

Analytical Data, WR5/BP 96-2 (Chlor) Chloroquine, Didesethylchloroquine and Monodesethylchloroquine Concentrations in Human Plasma and Blood (Continued)

Sample ID	Plasma CHLORO-QUINE (ng/ml)	Plasma DI-CHLORO (ng/ml)	Plasma MONO-CHLORO (ng/ml)	Blood CHLORO-QUINE (ng/ml)	Blood DI-CHLORO (ng/ml)	Blood MONO-CHLORO (ng/ml)
73-D(7/01/05)	36.5	31.8	75.0	73.9	41.6	80.4
73-D(7/01/19)	*	*	*	31.9	20.2	32.2
73-D(7/02/02)	*	*	*	*	*	*
73-D(7/02/15)	*	*	*	*	*	*
73-D(7/03/16)	*	*	*	*	*	*
73-D(7/04/15)	*	*	*	*	*	*
73-D(7/05/11)	*	*	*	*	*	*
74-A(6/12/22)	227	33.6	96.6	478	40.5	124
74-A(6/12/23)	158	22.0	65.4	404	40.8	118
74-A(6/12/24)	159	26.4	79.4	337	33.3	85.7
74-A(6/12/28)	62.4	21.3	53.5	158	25.2	48.9
74-A(7/01/02)	23.3	*	27.5	58.6	*	24.9

* = below assay sensitivity (20.0 ng/ml)

Art/P 00-1

Analytical Data, Art/P 00-1, Artelinic Acid Concentrations in Human Plasma

Final results for the "Inter-laboratory validation samples for artelinic acid measurement."

Label	Spiked Conc. (ng/ml)	Measured Conc. (ng/ml)
AL11	500	360
AL12	500	372
AL13	500	349
AL21	250	190
AL22	250	188
AL23	250	189
AL31	25	20.8
AL32	25	18.4
AL33	25	20.5
DFP	0	*